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TITLE: Pharmacological and Behavioral Enhancement of Neuroplasticity in the MPTP-Lesioned Mouse and Nonhuman Primate

PRINCIPAL INVESTIGATOR: Giselle Petzinger, M.D.
Michael W. Jakowec, Ph.D.

CONTRACTING ORGANIZATION: University of Southern California
Los Angeles, CA 90089-1147

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Giselle Petzinger, M.D. and Michael W. Jakowec, Ph.D.

E-Mail: gpetzinger@Surgery.usc.edu

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The purpose of this proposal is to investigate the mechanisms involving pharmacological and behavioral enhanced neuroplasticity of the injured basal ganglia. Our central hypothesis is that exercise and pharmacological intervention, specifically the administration of a D2 dopamine-receptor agonist, enhances neuroplasticity by modulating glutamate-dopamine interactions. This proposal has two components. Using the MPTP C57BL/6 mouse Component One will test the hypothesis that exercise enhances plasticity of the MPTP-injured basal ganglia through glutamate by modulating dopamine biosynthesis. This hypothesis will be tested through changes in dopamine, and proteins involved in dopamine biosynthesis and uptake (tyrosine hydroxylase and dopamine transporter) and changes in glutamatergic synapses and receptor subtype. This hypothesis will be tested through determining whether exercise-enhanced neuroplasticity may be attenuated with a glutamate antagonist. Using the MPTP-lesioned non-human primate Component Two will test the hypothesis that the D2 receptor agonist (Pramipexole) enhances neuroplasticity of the MPTP-injured basal ganglia through its effect on pre- and post- synaptic dopamine biosynthesis, uptake and receptor expression as well as glutamatergic synapses. This hypothesis will be tested through changes in dopamine and its metabolites, proteins involved in dopamine biosynthesis, uptake, and storage (tyrosine hydroxylase, dopamine transporter, and vesicular monoamine transporter), changes in dopamine receptor subtypes and their respective neuropeptides, and changes in glutamatergic synapses.

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## Introduction

The primary focus of this research proposal is to determine the underlying mechanisms responsible for neuroplasticity in the injured adult basal ganglia. For these studies we utilize the neurotoxicant MPTP that selectively destroys nigrostriatal dopaminergic neurons and leads to the depletion of striatal dopamine as well as the development of parkinsonian features. In the squirrel monkey these features include slowness of movement, balance impairment and diminished hand dexterity. In our laboratory we utilize both the MPTP-lesioned C57BL6 mouse and the MPTP-lesioned squirrel monkey. Both models show intrinsic plasticity through either striatal dopamine return (mouse) and/or behavioral recovery (squirrel monkey). In this proposal we were particularly interested in understanding whether exercise (mouse) or dopamine replacement therapy (monkey) might enhance intrinsic neuroplasticity of the injured basal ganglia. For this purpose, the proposal was divided into two components, a mouse exercise study and a squirrel monkey dopamine replacement study. These studies were designed to be complementary in that both nonpharmacological and pharmacological effects of neuroplasticity are being investigated.

In the following sections are included the abstract, introduction and specific aims from the original proposal. This is followed by the accomplishments and research outcomes from year one. This annual report also includes manuscripts in the form of appendices.


**Abstract** (From the Original Application)

The purpose of this proposal is to investigate the molecular mechanisms involving pharmacological and behavioral (exercise) enhanced neuroplasticity of the injured basal ganglia. Our central hypothesis is that exercise and pharmacological intervention, specifically the administration of a D2 dopamine-receptor agonist, enhances neuroplasticity by modulating glutamate-dopamine interactions. The following proposal has two complementary components using two animal models to address the molecular mechanisms underlying exercise- and pharmacologically-enhanced neuroplasticity. Using the MPTP C57BL/6 mouse **Component One** will test the hypothesis that exercise enhances plasticity of the MPTP-injured basal ganglia through glutamate by modulating dopamine biosynthesis. This hypothesis will be tested through changes in dopamine, and proteins involved in dopamine biosynthesis and uptake (tyrosine hydroxylase and dopamine transporter) and changes in glutamatergic synapses and receptor subtype. This hypothesis will be further tested through determining whether exercise-enhanced neuroplasticity may be attenuated with a glutamate antagonist. Using the MPTP-lesioned non-human primate **Component Two** will test the hypothesis that the administration of a D2 receptor agonist (Pramipexole) enhances neuroplasticity of the MPTP-injured basal ganglia through its effect on pre- and post- synaptic dopamine biosynthesis, uptake and receptor expression as well as glutamatergic synapses. This hypothesis will be tested through changes in dopamine and its metabolites, proteins involved in dopamine biosynthesis, uptake, and storage (tyrosine hydroxylase, dopamine transporter, and vesicular monoamine transporter), changes in dopamine receptor subtypes and their respective neuropeptides, and changes in glutamatergic synapses. By elucidating the role of exercise and pharmacological manipulation in neuroplasticity of the injured brain we hope to identify novel therapeutic targets for the treatment of brain injury and neurotoxic insult. Since military personnel are at risk for a wide range of brain injuries including head trauma, neurotoxic exposure (from pesticides, hostile enemy poisoning, viral and biological weapon based agents) it is imperative that medical strategies be made available to reverse the debilitating neurological deficits.


## D: STATEMENT OF WORK (From the original Application)

The brain's capacity for recovery from damage is far greater than previously recognized. It is now understood that neuroplasticity can be modulated through activity-dependent processes including exercise and environmental enrichment, and through pharmacological manipulation. Most of our understanding of exercise and pharmacological enhanced neuroplasticity is derived from studies in the cortex and the hippocampus, but there is mounting evidence that the same phenomenon occurs in the injured basal

ganglia. The molecular mechanisms for this phenomenon are not well understood. Using two animal models of injury induced neuroplasticity in the basal ganglia (the MPTP-lesioned mouse and MPTP-lesioned non-human primate) we propose to examine two modes of intervention to enhance neuroplasticity. These include exercise in the MPTP-lesioned mouse model and pharmacological intervention in the MPTP-lesioned non-human primate. Our central hypothesis is that exercise and pharmacological intervention, specifically the administration of a D2 dopamine-receptor agonist, enhances neuroplasticity by modulating glutamate-dopamine interactions.

The following proposal has two complementary components using both animal models to address the molecular mechanisms underlying exercise- and pharmacologically-enhanced neuroplasticity. Using the MPTP C57BL/6 mouse **Component One** will test the hypothesis that exercise enhances plasticity of the MPTP-injured basal ganglia through glutamate by modulating dopamine biosynthesis. This hypothesis will be tested through changes in dopamine, and proteins involved in dopamine biosynthesis and uptake (tyrosine hydroxylase and dopamine transporter) and changes in glutamatergic synapses and receptor subtype. This hypothesis will be further tested through determining whether exercise-enhanced neuroplasticity may be attenuated with a glutamate antagonist. Using the MPTP-lesioned non-human primate **Component Two** will test the hypothesis that the administration of a D2 receptor agonist (pramipexole) enhances neuroplasticity of the MPTP-injured basal ganglia through its effect on pre- and post- synaptic dopamine biosynthesis, uptake and receptor expression as well as glutamatergic synapses. This hypothesis will be tested through changes in dopamine and its metabolites, proteins involved in dopamine biosynthesis, uptake, and storage (tyrosine hydroxylase, dopamine transporter, and vesicular monoamine transporter), changes in dopamine receptor subtypes and their respective neuropeptides, and changes in glutamatergic synapses. By elucidating the role of exercise and pharmacological manipulation in neuroplasticity of the injured brain we will identify new therapeutic targets for the treatment of traumatic brain injury and neurotoxic insult, two high-risk morbidities that are common to military personnel.

**<u>Component One</u>: To test the hypothesis that exercise enhances neuroplasticity of the MPTP-lesioned mouse through glutamate by modulating dopamine biosynthesis.**

Component One will utilize the following 4 treatment groups for **Study1** through **Study 4**:

(1) Saline-injected;
(2) MPTP-injected;
(3) Saline-injected + exercise;
(4) MPTP-injected + exercise.

**Study 5** will utilize the following glutamate antagonists: AMPA antagonist (GYKI-52466) and the NMDA antagonist (MK-801) in the following 8 treatment groups:

(1) Saline-injected + GYKI-52466;
(2) MPTP-injected + GYKI-52466;
(3) Saline-injected + exercise + GYKI-52466;
(4) MPTP-injected + exercise + GYKI-52466;
(5) Saline-injected + MK801;
(6) MPTP-injected + MK801;
(7) Saline-injected + exercise + MK801;
(8) MPTP-injected + exercise +MK801.

Exercise will be performed on a motorized rodent treadmill. Brain tissue will be collected after 30 days of running.

**Study 1**: The level of striatal dopamine and its metabolites will be determined using HPLC analysis comparing exercise versus non-exercise groups in the MPTP-lesioned mouse.

**Study 2**: The pattern of expression of striatal tyrosine hydroxylase (TH), dopamine transporter (DAT), cAMP-responsive enhancer binding protein (CREB), phospho~CREB, and dopamine- and adenosine- 3':5'-monophosphate-regulated phosphoprotein (DARPP-32), and phospho~DARPP-32 protein and their mRNA transcripts in surviving dopaminergic neurons will be determined using immunohistochemistry, western immunoblotting, *in situ* hybridization and correlated with striatal dopamine return. Pilot data shows attenuation of the return of DAT protein, and TH mRNA by exercise in MPTP-lesioned mice.

**Study 3:** The effect of exercise on glutamatergic synapses in the striatum after injury will be determined using ultrastructural immunohistochemical staining with electron microscopy. Pilot data shows altered glutamatergic synapses using immuno-electron microscopy.

**Study 4:** The pattern of expression of subunits for both the NMDA and AMPA receptor subtypes and their phosphorylated state will be determined using western immunoblotting, immunocytochemistry and *in situ* hybridization histochemistry.

**Study 5**: *We will test the hypothesis that exercise induced neuroplasticity can be attenuated through the administration of either a NMDA or AMPA receptor antagonist*. After MPTP-lesioning mice will be subjected to exercise while receiving either the NMDA receptor antagonist MK-801 or the AMPA receptor antagonist GYKI-52466. Behavioral recovery will be compared between groups. Brain tissue will be analyzed for alteration in dopaminergic function (dopamine, DAT and TH expression). Pilot studies show that both glutamate receptor antagonists GYKI-52466 and MK-801 can be administered in this model of MPTP-lesioning.

**Component Two: To test the hypothesis that the administration of a D2 receptor agonist (pramipexole) enhances neuroplasticity of the MPTP-lesioned non-human primate through its effect on dopamine (biosynthesis, uptake, and receptor expression) and glutamatergic synapses.**

Component Two will utilize the following treatment groups (n = 4 per group):

(1) Saline-injected harvested at 6 weeks after the last injection;
(2) Saline-injected harvested at 16 weeks after the last injection;
(3) MPTP-injected harvested at 6 weeks after the last injection;
(4) MPTP-injected harvested at 16 weeks after the last injection;
(5) Saline-injected + pramipexole harvested at 6 weeks after the last injection;
(6) Saline-injected + pramipexole harvested at 16 weeks after the last injection;
(7) MPTP-injected + pramipexole harvested at 6 weeks after the last injection;
(8) MPTP-injected + pramipexole harvested at 16 weeks after the last injection.

**Study 1:** The behavioral recovery of saline injected and MPTP-lesioned squirrel monkeys will be compared with and without the administration of pramipexole. Animal behavior will be monitored using both a cage side clinical rating scale and a personal activity monitor.

**Study 2:** The pattern of expression of proteins and mRNA transcripts important for dopaminergic function, (including TH, DAT, VMAT2) at the level of the SNpc and CPu will be determined. Preliminary data supports our ability to carry out western immunoblotting, immunocytochemistry and *in situ* hybridization in the MPTP-lesioned non-human primate.

**Study 3:** The pattern of expression of the dopamine receptors D1, D2, and D3 will be determined in both the SNpc and CPu. The level of protein expression will be determined western immunoblotting, immunohistochemistry, while the level of mRNA transcript expression will be determined using *in situ* hybridization histochemistry. Double labeling techniques will be used to co-localize the dopamine receptor changes with other enkephalin or substance P containing neurons. Preliminary data supports our ability to use these techniques in the non-human primate.

**Study 4:** The effect of pramipexole on glutamatergic synapses in the striatum after injury will be determined using ultrastructural immunohistochemical staining with electron microscopy. Pilot data shows our ability to quantify glutamatergic synapses using immuno-electron microscopy.

At the conclusion of these studies we will have a better understanding on the role of exercise and dopamine agonist (pramipexole) treatment in enhancing neuroplasticity of the injured basal ganglia in the mouse and the non-human primate. This may then identify important therapeutic targets (through glutamate and dopamine) for the treatment of brain injury.

**Table 1: Timeline of Experimental Design for Component One (Exercise in the MPTP-Lesioned Mouse Model).**

| Component One: Exercise in the MPTP-lesioned Mouse | | | | |
|---|---|---|---|---|
| | **Year 1** | **Year 2** | **Year 3** | **Year 4** |
| **Study 1:** Analysis of Dopamine and its metabolites | HPLC | | | |
| **Study 2:** Analysis of TH, DAT, CREB, and DARPP-32 | Immunocytochemistry, In Situ Hybridization, Western Immunoblotting | | | |
| **Study 3:** Analysis of striatal glutamate synapses | | Immuno-electron microscopy | | |
| **Study 4:** Analysis of NMDA and AMPA receptor subtypes | | Immunocytochemistry, In Situ Hybridization, Western Immunoblotting | | |
| **Study 5:** Attenuate neuroplasticity with NMDA and AMPA receptor antagonists | | | Immunocytochemistry, In Situ Hybridization, Western Immunoblotting, Immuno-electron microscopy | |

**<u>Component 2</u>: <u>Pharmacological Enhancement of Neuroplasticity in the MPTP-lesioned Non-Human Primate Model.</u>**

**Time Line:**

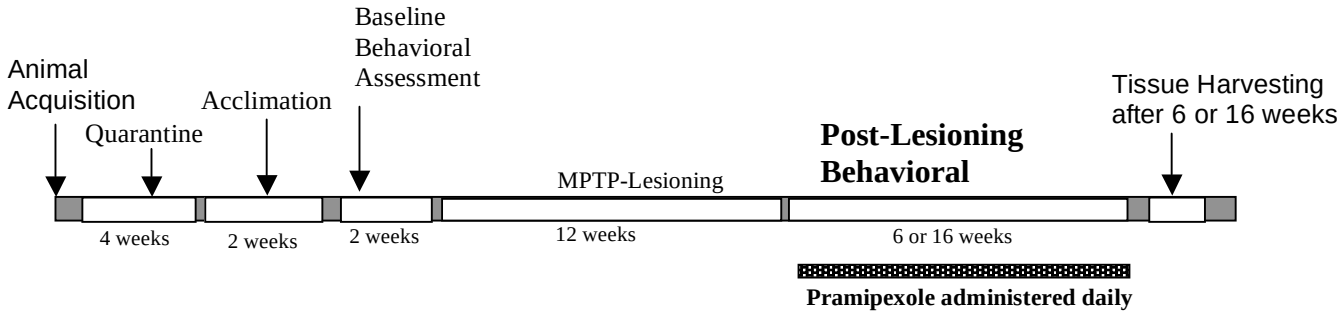



**Table 2: Timeline of Experimental Design for Component Two (MPTP-Lesioned Primate Model).**

| **Specific Aim** | **Year 1** | **Year 2** | **Year 3** | **Year 4** |
|---|---|---|---|---|
| | Lesion animals and administer Pramipexole | | | |
| Study 1: Behavior | Behavioral analysis | | | |
| Study 2: Molecular | | TH, DAT, VMAT mRNA and protein using WIB, ICC and ISH | | |
| Study 3: Dopamine Receptors | | Dopamine Receptor D1, D2, and D3 using WIB, ICC, and ISH | | |
| Study 4: Glutamate | | Analysis of glutamatergic synapses using immuno-EM | | |

## Key Research Accomplishments for Years One Through Three

**Component One: Enhancement of neuroplasticity in the MPTP-lesioned mouse**

(i) Intensive treadmill exercise leads to improved motor performance of both MPTP-lesioned and saline treated mice. Specifically exercised animals run faster and for a longer duration. This is published in Fisher et al 2004 and Petzinger et al 2007.

(ii) Analysis of behavior using the accelerating rotarod as a second measure shows that both saline and MPTP-lesioned mice demonstrate that these mice display increased level of motor learning compared to animals that have not gone through intensive treadmill exercise. This is published in Petzinger et al 2007.

(iii) Intensive treadmill exercise suppresses the intrinsic return of striatal dopamine transporter (DAT) protein. On further analysis tyrosine hydroxylase protein does not appear to be significantly altered by exercise in the MPTP-lesioned mouse. This is published in Fisher et al 2004 and Petzinger et al 2007.

(iv) Intensive treadmill exercise suppresses the expression of dopamine transporter mRNA transcripts in both saline + exercise and MPTP + exercise mice. On further analysis there does not appear to be a significant reduction for the tyrosine hydroxylase mRNA transcript after exercise.

(v) Intensive treadmill exercise causes a normalization of synaptic glutamate to levels seen in non-lesioned mice without exercise. This is published in Fisher et al 2004.

(vi) The administration of AMPA and NMDA receptor antagonists altered the pattern of expression of tyrosine hydroxylase and dopamine transporter mRNA transcription in nigrostriatal dopaminergic neurons as well as the pattern of expression of striatal tyrosine hydroxylase.

(vii) Electrophysiological analysis of dopamine release using fast-cyclic voltammetry indicates increased release of dopamine in the MPTP-lesioned mouse undergoing intensive treadmill exercise compared to MPTP-lesioned or saline. This is published in Petzinger et al 2007.

(vii) Intensive treadmill running increases the D2 receptor mRNA transcript expression but does not alter the expression of D1 receptor subtype. This is published in Fisher et al 2004.

(viii) There is no alteration in the number of SNpc neurons with exercise in either MPTP-lesioned or saline treated mice. This is published in Petzinger et al 2007.

(ix) Immunohistochemical staining with antibody probes against GluR1 and GluR2 and their phosphorylated state shows exercise dependent alterations in the degree and pattern of expression. Results are to be presented at the Society for Neuroscience Annual meeting San Diego, November 2007. A manuscript entitled “Altered AMPA-Receptor Expression with Treadmill Exercise in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Lesioned Mouse Model of Basal Ganglia Injury”.

(x) Analysis of mRNA transcripts for the AMPA receptors GluR1 through GluR4 (including their flip and flop isoforms) and the NMDA receptors NR1, NR2A through 2D has been carried out. Results indicate differential expression of some subunits in response to exercise or MPTP-lesioning. Some of these data are presented in this report and are also are to be presented at the Society for Neuroscience Annual meeting San Diego, November 2007. A manuscript entitled “Altered AMPA-Receptor Expression with Treadmill Exercise in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Lesioned Mouse Model of Basal Ganglia Injury”.

(xi) Electrophysiological analysis of the relative contribution of AMPA and NMDA receptors in the conductance of currents within the striatum show that there are changes that are exercise dependent. Alterations in LTP and LTD and the ratio of AMPA to NMDA receptor expression with exercise in the MPTP-lesioned mouse model will be completed in year 4 of this proposal.

(xii) Golgi staining shows some differences in the density of dendritic spines but we are unable to differentiate between the direct population of striatal projection neurons (dopamine receptor D1 containing) and the indirect population (expressing the dopamine receptor D2) using this technique. We have initiated an alternative approach to delineate these populations and analyze the density of dendritic spines with exercise in both saline and MPTP-lesioned mice.

**Component Two: Enhancement of neuroplasticity in the MPTP-lesioned nonhuman primate.**

(i) The administration of the dopamine agonist Pramipexole induces dyskinesia. This occurs to a lesser degree than that observed with Sinemet.

(ii) Pramipexole and Sinemet increases dopamine levels in both the MPTP-lesioned mouse and MPTP-lesioned squirrel monkey. These changes are most evident in the ventral striatum.

(ii) The addition of microdialysis studies supports the neurochemical HPLC analysis. Specifically treated animals that have undergone repeated microdialysis studies demonstrate an increase in amphetamine induced dopamine release after termination of either Sinemet or Pramipexole treatment.

(iv) Western blot analysis demonstrates a slight increase in TH and DAT protein expression in the dorsal caudate in Pramipexole-treated animals, but no change in DAT expression. Studies examining subregions of the striatum for changes in TH, DAT, DARPP-32, and VMAT protein expression both by western and immunocytochemistry techniques show altered patterns of expression in animals receiving dopamine replacement therapy.

(v) Neurophysiological studies show no difference in dopamine release using fast-scan cyclic voltammetry. Methodological differences between microdialysis and voltammetry may explain why no increase in dopamine was observed in the drug treated groups using voltammetry.

(vi) Neurophysiological studies have demonstrated a low AMPA to NMDA ratio in normal medium spiny neurons of the nonhuman primates. After MPTP lesioning this ratio appears to diverge into two distinct AMPA/NMDA ratio characteristics. In general the population of medium spiny cells appear to diverge either to increase AMPA to NMDA ratio and another medium spiny cell type decreases AMPA to NMDA ration. We found that the input/output relationship was greater for AMPA receptor mediated synaptic currents at 6 weeks after MPTP-lesioning compared to saline control using whole cell voltage clamp. Analysis of animals 9 months after MPTP administration suggests there is normalization of corticostriatal hyperactivity when animals demonstrate full behavioral recovery.Also, that LTD expression at lateral cortico-putamen synapses from the 9-month MPTP-lesioned squirrel monkey is D2 dependent. These neurophysiological studies have been added as an important and informative deviation from the original proposal outline.

(vii) MPTP-lesioned animals demonstrate increased glutamate terminal density relative to saline treated animals. After treatment with either Sinemet or Pramipexole, there is reduced glutamate terminal density occupancy. This finding would support but not confirm that drug treatment facilitates the release of glutamate within corticostriatal terminals. Given the presence of dyskinesia in these treated animals this finding may also support the hypothesis that increased dyskinesia may be in part related to glutamate release.

# Key Research Accomplishments for Year Three

One of the key aims of this proposal is to elucidate the mechanisms responsible for improved motor behavior in the MPTP-lesioned mouse model of basal ganglia injury. In our previous report (Fisher et al 2004) studies using immuno-electron microscopy showed that there were alterations in the synaptic occupancy of glutamate such that the MPTP-induced increase is normalized by intensive treadmill exercise. In addition, ongoing electrophysiological studies examining the changes in glutamate receptor expression through pharmacological analysis in *in vitro* slice culture have indicated alterations in the ration of AMPA to NMDA receptor composition of medium spiny neurons as well as a shift in the subunit specific makeup of receptors. To examine the basis of this shift in subunit composition we have carried out an analysis of the pattern of expression of two key AMPA receptor subunits GluR1 and GluR2. The analysis of these subunits is also influenced by our recent findings showing that there is a shift between long-term potentiation and long-term depression with exercise in the MPTP-lesioned mouse undergoing intensive treadmill exercise. The following section describes alterations in the pattern of expression of AMPA receptor subunits GluR1 and GluR2 mRNA transcripts and their flip and flop isoforms using quantitative real-time PCR as well as their protein expression patterns including phosphorylated states using immunohistochemical immuno-staining. These analyses focused on the dorsolateral striatum, a region responsible for motor control.

**Results**

***Treadmill Running Behavior***

The time course of improvement in running velocity of both the saline+exercise and MPTP+exercise groups over the 6 weeks (28 days) of treadmill running is shown in Figure 1. Saline+exercise mice in the first week of treadmill running started at a velocity of 14 ± 1.4 m/min that further increased to 22.6 ± 0.3 m/min by the final week. The MPTP+exercise group had a running velocity of 9.2 ± 1.1 m/min during the first week that further increased to 20.5 ± 0.7 m/min in the last week. As shown in our previous papers, there was a significant difference in velocity at week 1 between the saline+exercise and MPTP+exercise groups and this difference was not significant at the completion of the treadmill running regimen [Fisher, 2004; Petzinger 2007).

***HPLC Analysis of Striatal Dopamine***

HPLC analysis was used to determine levels of striatal dopamine, its metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), and the metabolites turnover ratio, defined as [(DOPAC + HVA)/dopamine]. These data are shown in Table 2. To determine the degree of dopamine depletion by MPTP-lesioning, a subset of non-exercised mice from the saline and MPTP-lesioned groups was analyzed 10 days post-lesioning. At the 10-day time point, the MPTP (48.0 ± 8.4 ng dopamine/mg protein) mice showed significantly lower levels of striatal dopamine compared to the saline group (269.5 ± 24.9 ng dopamine/mg protein) ($p < 0.05$), which represented an 83% depletion. Analysis of dopamine turnover ratio showed a significant elevation in the MPTP group (turnover ratio = 2.3), at the 10-day time point, compared to the saline group (turnover ratio = 0.3) ($p < 0.05$).

HPLC analysis of striatal dopamine at the completion of the 28 days of treadmill running (42 days post-MPTP lesioning) showed that dopamine levels remained significantly depleted in MPTP-lesioned mice compared to saline controls ($F = 229.3$, $p < 0.0001$). There was no significant difference in striatal dopamine levels comparing MPTP+exercise with MPTP sedentary mice. There was a significant effect of exercise on the saline treated group, where saline+exercise mice had a higher level of striatal dopamine compared to saline mice ($F = 7.78$, $p = 0.015$). There were no significant effects of MPTP or exercise, or interaction between these two factors on turnover ratio, with the ratios of MPTP = 0.36, MPTP+exercise = 0.34, saline = 0.26, and saline+exercise group = 0.34.

**Analysis of AMPA Receptor Subunits GluR1 and GluR2 Expression**

The pattern of expression of mRNA transcripts and proteins for the AMPA receptor subunits GluR1 and GluR2 were determined using quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemical staining, respectively. The design of primer sets for qRT-PCR also allowed for the

analysis of the common isoforms of both GluR1 and GluR2 due to alternative transcript splicing, termed flip and flop. In addition, immunohistochemical staining for AMPA-R subunits was determined using antibodies that recognized either the pan or phosphorylated forms of GluR1 and GluR2.

***Receptor Subunit GluR1***

Immunohistochemical staining of GluR1 subunit expression using a pan-specific antibody shown in Figure 2 showed that the total number of immunoreactive-positive cells decreased in the dorsolateral striatum of the MPTP-lesioned group compared with saline mice, but did not quite reach statistical significance ($F = 4.852$; $p = 0.0587$). There was no significant effect with exercise ($F = 0.04$; $p = 0.846$) and no interaction ($F = 0.063$; $p = 0.809$); specifically, there were no significant differences between saline and saline+exercise groups or the MPTP and MPTP+exercise groups. Analysis of GluR1-immunoreactivity within the neuropil of the dorsolateral striatum showed no significant differences in optical density between the saline and MPTP-lesioned groups ($F = 1.599$; $p = 0.242$) and between the exercise and non-exercise groups ($F = 1.247$; $p = 0.297$), and there was no interaction ($F = 0.905$; $p = 0.369$).

Immunohistochemical staining for the phosphorylated form of GluR1 was carried out using an antibody that recognizes GuR1~phospho-Ser845 (see Figure 3). There were no significant differences seen in the number of immuno-positive cells between the MPTP-lesioned and saline groups ($F = 0.918$; $p = 0.352$) nor between the exercise and non-exercise groups ($F = 1.493$; $p = 0.239$), and there was no interaction ($F = 0.113$; $p = 0.742$). Analysis of GuR1~phospho-Ser845 immunoreactivity within the neuropil of the dorsolateral striatum showed no significant differences in optical density between the saline and MPTP-lesioned groups ($F = 1.0$; $p = 0.329$) and between the exercise and non-exercise groups ($F = 0.999$; $p = 0.329$), and there was no interaction ($F = 1.0$; $p = 0.329$).

There was an apparent difference in the intensity of cell body immuno-staining between the different treatment groups within the dorsolateral striatum. Optical density measurements of cell body immunoreactivity captured at high magnification showed no significant difference between MPTP-lesioned mice compared to saline mice ($F = 0.029$ ; $p = 0.866$), and no significant difference between exercise and non-exercise mice ($F = 1.564$; $p = 0.226$). There was a significant interaction between treatment and exercise ($F = 6.728$; $p = 0.017$), such that exercise led to decreased expression of GluR1~phospho-Ser 845 immunoreactivity within cell bodies of saline treated mice and an increase in expression in cell bodies of MPTP-lesioned mice.

Analysis of mRNA transcript for the pan-GluR1 within the dorsolateral striatum (see Figure 4A) showed that there was a significant decrease in the expression of GluR1 in MPTP-lesioned compared with saline treated mice ($F = 444.0$; $p < 0.0001$) and a significant decrease in the expression of GluR1 in exercise compared to non-exercise mice ($F = 159.0$; $p < 0.0001$). In addition there was a significant interaction between treatment and exercise ($F = 135.9$; $p <0.0001$), such that exercise led to a decrease expression of GluR1 in the saline group.

Analysis of mRNA transcript for the flip (see Figure 4B) and flop (see Figure 4C) isoforms of GluR1 within the dorsolateral striatum showed an altered pattern of expression. Specifically, there was a significant decrease in the expression of GluR1-flip in the exercise compared to non-exercise groups ($F = 52.05$; $p < 0.0001$). There was no significant effect of MPTP-lesioning on GluR1-flip expression ($F = 0.640$; $p = 0.447$) and no interaction between exercise and MPTP-lesioning ($F = 0.0002$; $p = 0.989$). With GluR1-flop there was a significant decrease in the MPTP-lesioned group compared to saline ($F = 103.3$; $p < 0.0001$). There was no significant effect of exercise on GluR1-flop expression ($F = 3.646$; $p = 0.093$). There was a slight trend for an interaction between exercise and MPTP-lesioning ($F = 3.979$, $p = 0.081$), due to an increased expression in GluR1-flop with exercise in the saline group.

**Receptor Subunit GluR2**

Immunohistochemical staining of GluR2 subunit expression using a pan-specific antibody shown in Figure 5 showed that the total number of immunoreactive-positive cells increased in the dorsolateral striatum of the MPTP-lesioned group compared with saline mice ($F = 10.370$; $p = 0.012$). There was no significant effect with exercise ($F = 1.133$; $p = 0.318$). There was a trend towards significance in the

interaction between exercise and MPTP-lesioning ($F = 4.083$; $p = 0.078$). Specifically, we observed an increase in the expression of GluR2-immuno-reactivity in the MPTP+exercise mice compared to the MPTP non-exercise mice. Analysis of GluR2-immunoreactivity within the neuropil of the dorsolateral striatum showed no significant differences in optical density between the saline and MPTP-lesioned groups ($F = 0.358$; $p = 0.566$) and between the exercise and non-exercise groups ($F = 0.178$; $p = 0.684$), and there was no interaction ($F = 0.564$; $p = 0.474$).

In addition to an increase in the number of immunoreactive cells, we also observed morphological changes (degree of arborization) in cells expressing GluR2 (See Figure 5). Specifically, there was a decrease in the arborization of GluR2 immunoreactive cell bodies in MPTP-lesioned compared with saline mice. Interestingly, we observed a dramatic increase in arborization in saline + exercise compared with saline non-exercise mice.

Immunohistochemical staining for the phosphorylated form of GluR2 was carried out using an antibody that recognizes GuR2~phospho-Ser880 (see Figure 6). There was no significant difference in the number of immuno-positive cells between the MPTP-lesioned and saline groups ($F = 2.136$; $p = 0.182$). There was a significant effect with exercise ($F = 19.22$; $p < 0.002$) and there was a significant interaction between exercise and MPTP-lesioning ($F = 5.805$; $p < 0.043$). Specifically, we observed an increase in the expression of immuno-reactivity in the saline+exercise mice compared to the saline non-exercise mice. Analysis of GuR2~phospho-Ser880 immunoreactivity within the neuropil of the dorsolateral striatum showed no significant differences in optical density between the saline and MPTP-lesioned groups ($F = 2.345$; $p = 0.164$) and between the exercise and non-exercise groups ($F = 0.461$; $p = 0.516$), and there was no interaction ($F = 2.245$; $p = 0.172$). In addition to an increase in the number of immunoreactive cells, we also observed changes in the pattern of immunoreactivity within cell bodies expressing GuR2~phospho-Ser880 between the exercise and non-exercise groups (See Figure 6). The non-exercise group showed more homogeneous staining and the exercise group showed predominant staining in the perimeter of the cell body.

Analysis of mRNA transcript for the pan-GluR2 within the dorsolateral striatum (see Figure 7A) showed that there was a significant decrease in the expression of pan-GluR2 in MPTP-lesioned compared with saline treated mice ($F = 14.83$; $p < 0.005$) and a significant decrease in the expression of pan-GluR2 in exercise compared to non-exercise mice ($F = 9.180$; $p < 0.016$). There was no significant interaction between treatment and exercise ($F = 1.224$; $p = 0.301$).

Analysis of mRNA transcript for the GluR2-flip (see Figure 7B) and GluR2-flop (see Figure 7C) isoforms of GluR2 within the dorsolateral striatum showed an altered pattern of expression. Specifically, there was a significant decrease in the expression of GluR2-flip in the exercise compared to non-exercise groups ($F = 35.90$; $p < 0.0003$). There was also a significant decrease in the expression of GluR2-flip in the MPTP-lesioned group compared to the saline group ($F = 22.47$; $p < 0.0015$). There was no interaction between exercise and MPTP-lesioning ($F = 1.542$; $p = 0.25$). With GluR2-flop there was a significant increase in the MPTP-lesioned group compared to saline ($F = 55.71$; $p < 0.0001$). There was no significant effect of exercise on GluR2-flop expression ($F = 0.04$; $p = 0.843$) and no interaction between exercise and MPTP-lesioning ($F = 2.935$; $p = 0.125$).

**DARPP-32**

Immunohistochemical staining of DARPP-32 expression was carried out using antibodies that recognize either the pan-specific isoforms (see Figure 8) or the phospho-Thr75 isoforms (see Figure 9). Analysis of DARPP-32 with a pan-specific antibody demonstrated that there was no significant change in the number of immuno-positive cells in MPTP-lesioned group compared to the saline group ($F = 1.334$; $p = .286$). There was a decrease in animals that underwent intensive treadmill exercise compared to non-exercised animals (see Figure 8). This change, however, did not reach statistical significance ($F = 2.47$; $p = 0.16$). There was no significant interaction between exercise and MPTP-lesioning ($F = 0.002$; $p = 0.97$). Further analysis using the antibody specific for the phospho-Thr75 isoform of DARPP-32 demonstrated no significant change in the number of immuno-positive cells in the MPTP-lesioned group compared to saline

animals (F = 0.315; p = 0.590), no significant change in the exercise compared to the non-exercise group (F = 0.167; p = 0.693), and no significant interaction (F = 1.599; p = 0.242).

Table 1: PCR Primer sets used for analysis of AMPA receptor subunits and their alternative splice variants.

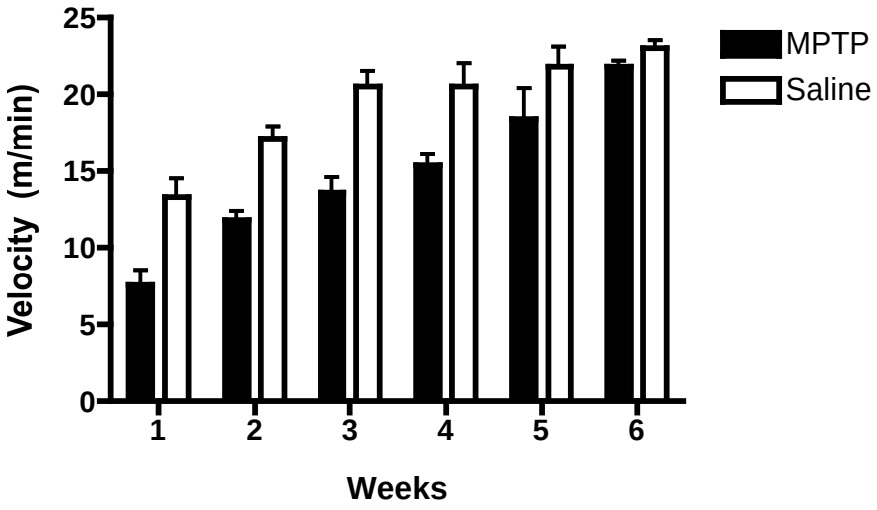
**Primer Sequences for AMPA-R Subunits and isoforms**

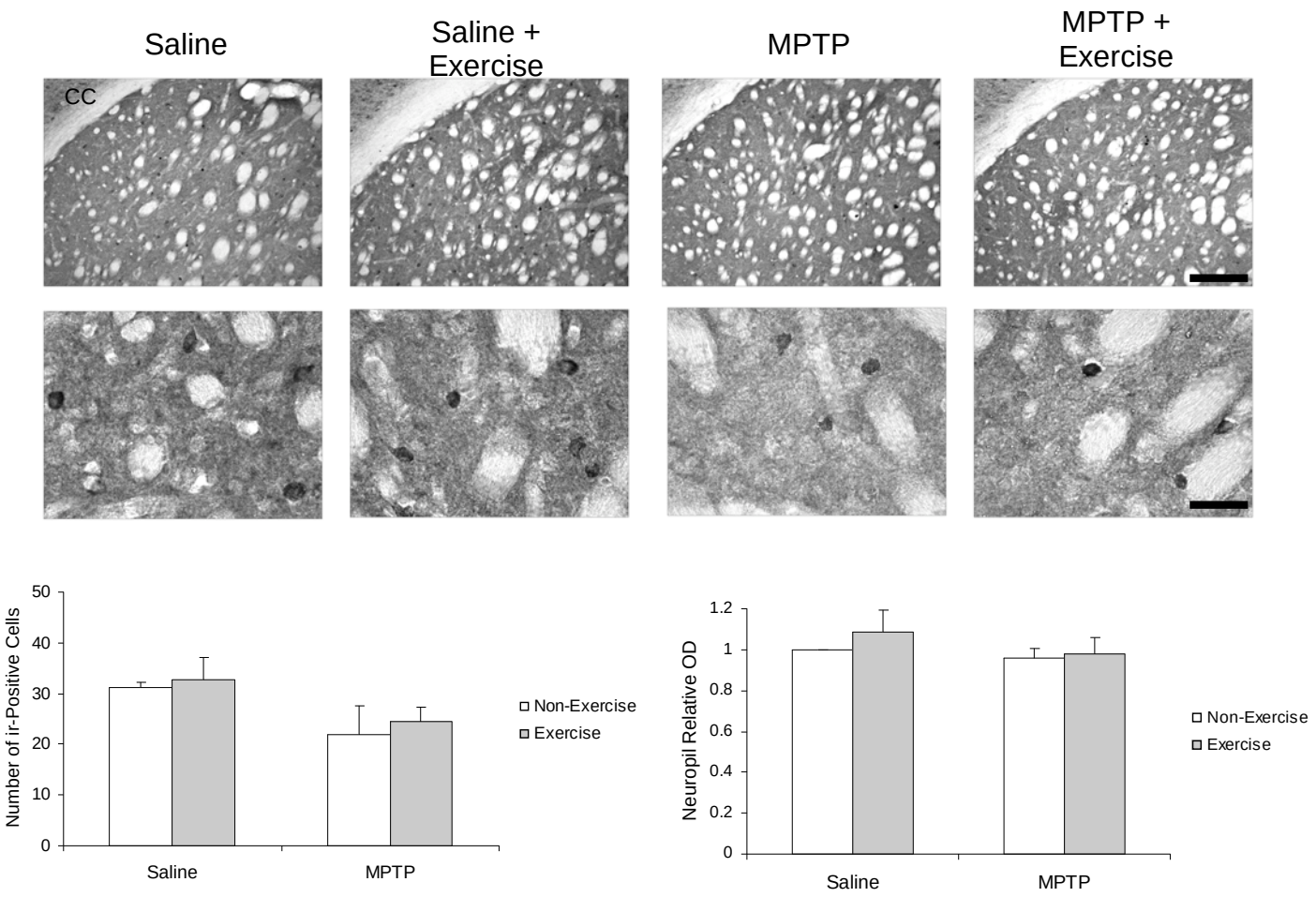
| Subunit | Species | Nucleotide sequence (5'-3') |
|---|---|---|
| GluR1 (F) | Mouse | ACA CCA TGA AAG TGG GAG GTA ACT |
| GluR1-flip (R) | | ACT GGT CTT GTC CTT ACT TCC GGA |
| GluR1-flop (R) | | ACT GGT CTT GTC CTT GGA GTC ACC |
| GluR2 (F) | Mouse | ACA CCA TGA AAG TGG GCG GCA ACC |
| GluR2-flip (R) | | ACT GGT CTT TTC CTT ACT TCC CGA |
| GluR2-flop (R) | | ACT GGT CTT TTC CTT GGA ATC ACC |
| Control | Mouse | |
| GAPDH (F) | | TGC ACC ACC AAC TGC TTA G |
| GAPDH (R) | | GGA TGC AGG GAT GAT GTT GTTC |

Table 2: HPLC analysis of striatal dopamine and its metabolites.

| | | | Dopamine | DOPAC | HVA | Turnover |
|---|---|---|---|---|---|---|
| Day 10 Post-MPTP | No Exercise | Saline | 269.5 ± 24.9 | 33.4 ± 4.5 | 33.1 ± 3.2 | 0.3 ± 0.08 |
| | | MPTP | 48.0 ± 8.4* | 11.7 ± 1.9* | 87.2 ± 9.6 | 2.3 ± 0.28* |
| Day 42 Post-MPTP | No Exercise | Saline | 246.9 ± 19.8 | 36.5 ± 4.5 | 27.4 ± 1.7 | 0.3 ± 0.01 |
| | | MPTP | 77.9 ± 12.0* | 14.7 ± 2.4* | 13.5 ± 1.9* | 0.4 ± 0.01 |
| | Exercise (28 days) | Saline | 315.2 ± 9.0$^{+}$ | 39.3 ± 2.8 | 25.9 ± 1.3 | 0.2 ± 0.01 |
| | | MPTP | 69.8 ± 11.7* | 11.3 ± 1.5* | 13.5 ± 1.2* | 0.4 ± 0.05 |

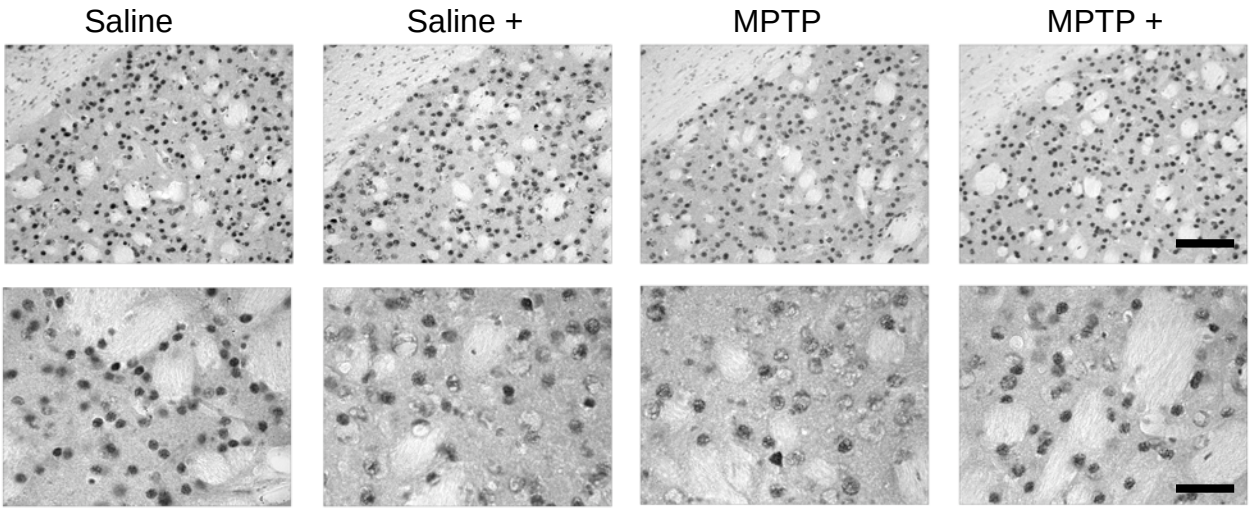
Figure 1:  Analysis of treadmill velocity running in both saline and MPTP-lesioned mice. Each point is an average for each week.

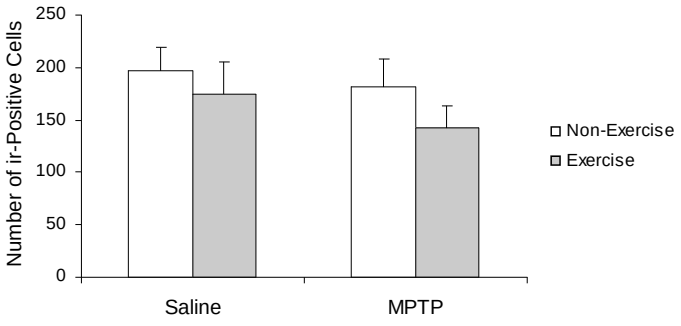


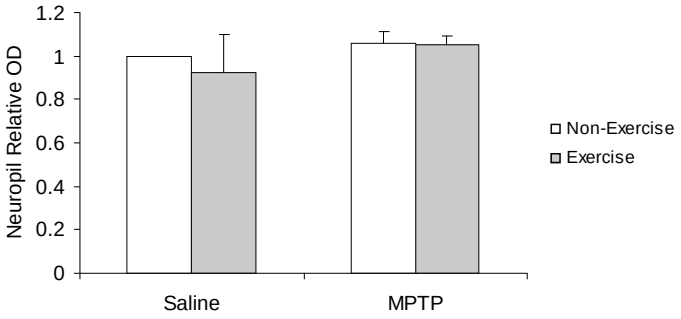


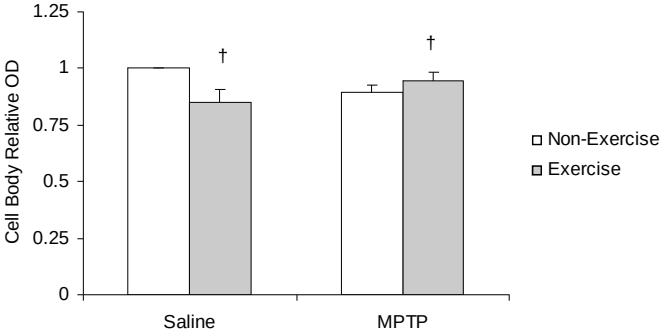

# GluR1

Figure 2: Immunohistochemical staining for the AMPA receptor subunit GluR1 in the dorsolateral striatum.



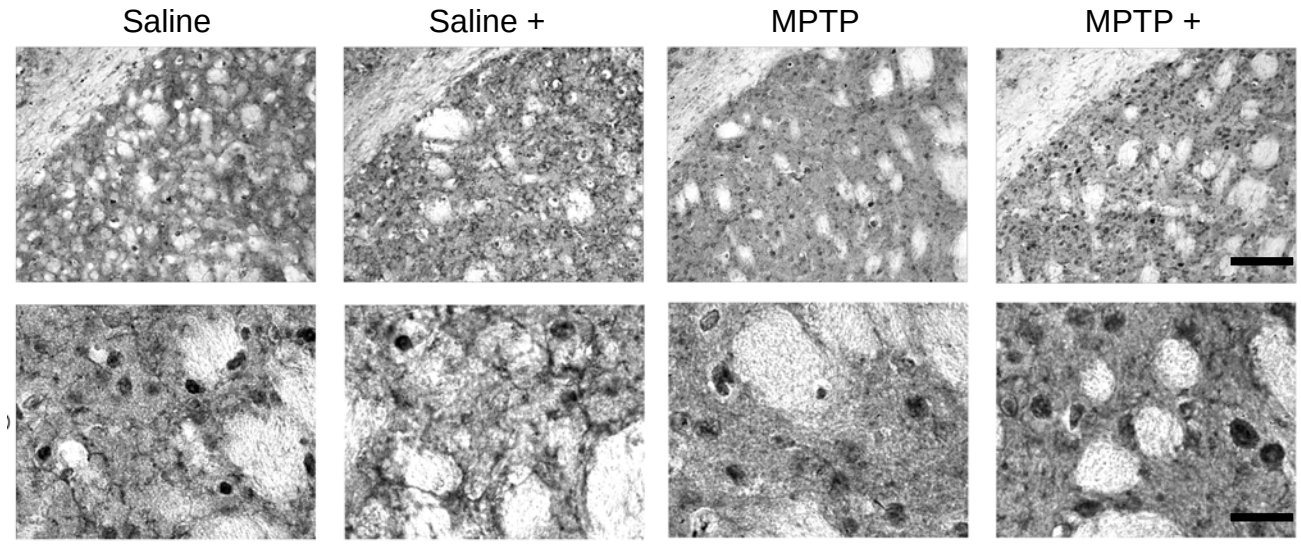



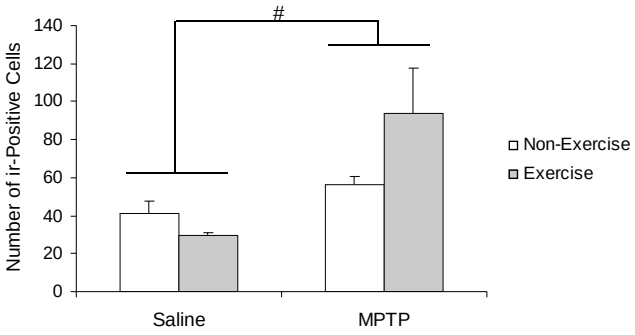




## GluR1 Ser845

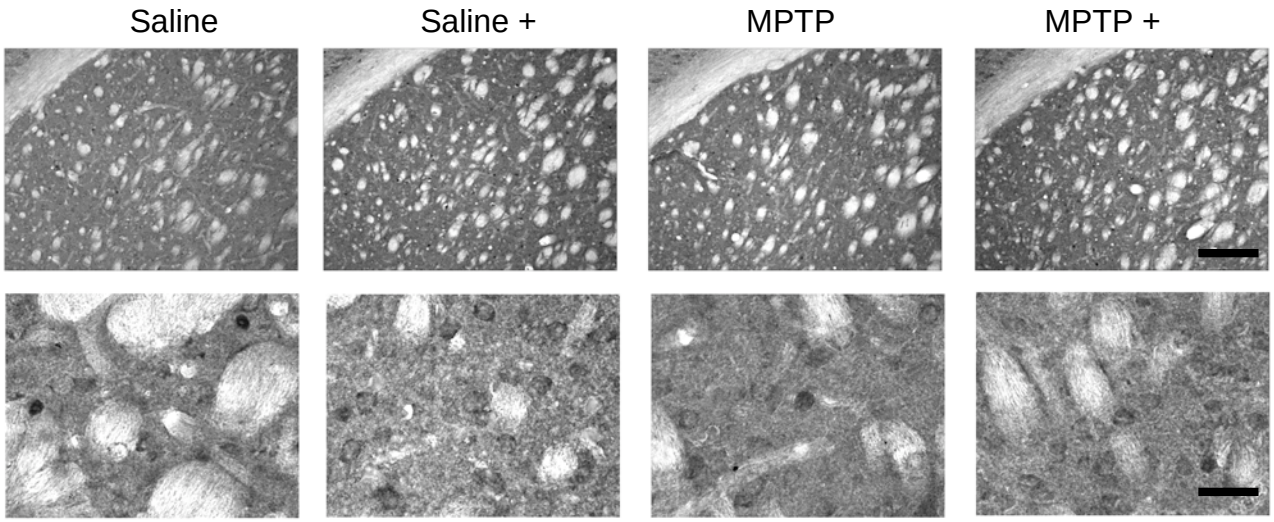
Figure 3; Immunohistochemical staining for the AMPA receptor subunit GluR1 phosphorylated at position Serine-845 in the dorsolateral striatum.

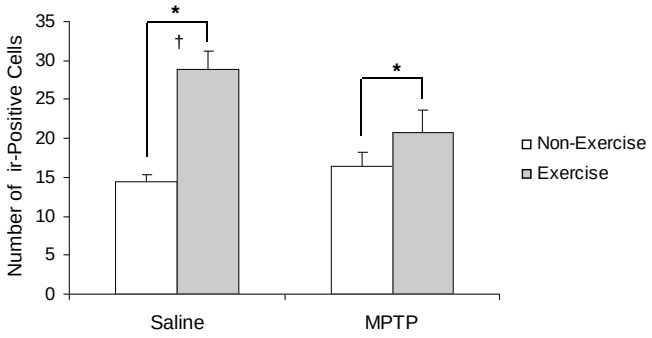



## GluR2

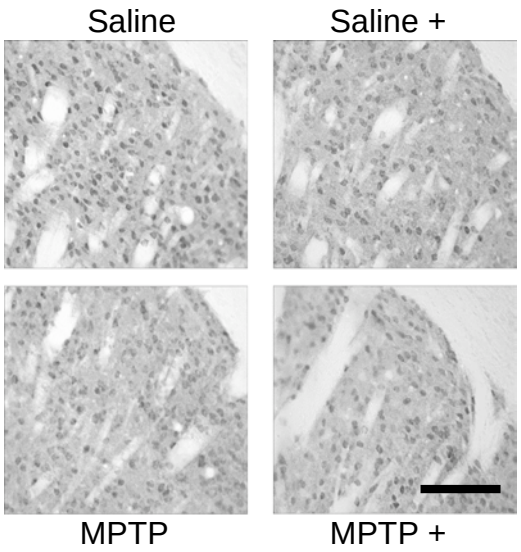
Figure 5: Immunohistochemical staining for the AMPA receptor subunit GluR1 in the dorsolateral striatum.

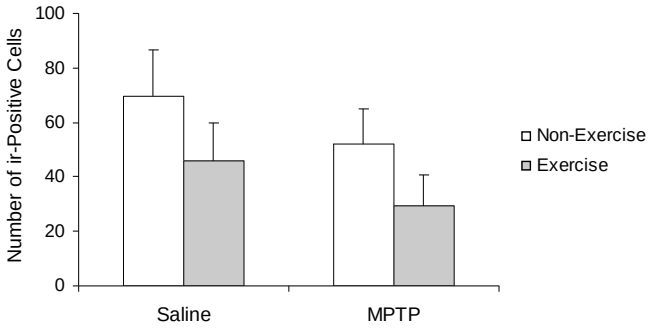



**GluR2 Ser880**

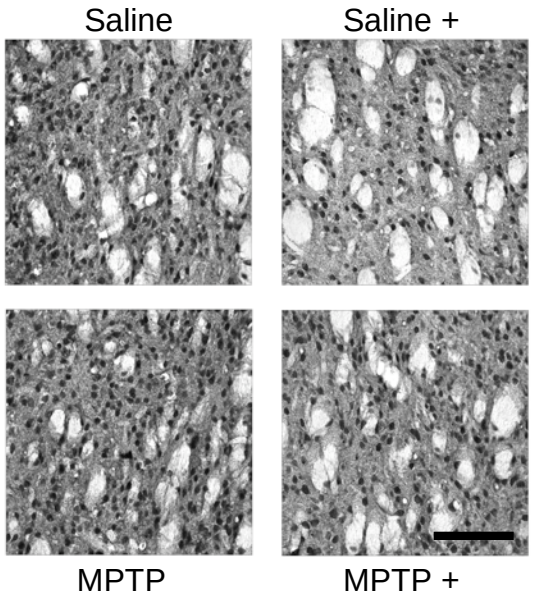
Figure 6: Immunohistochemical staining for the AMPA receptor subunit GluR1 in the dorsolateral striatum.

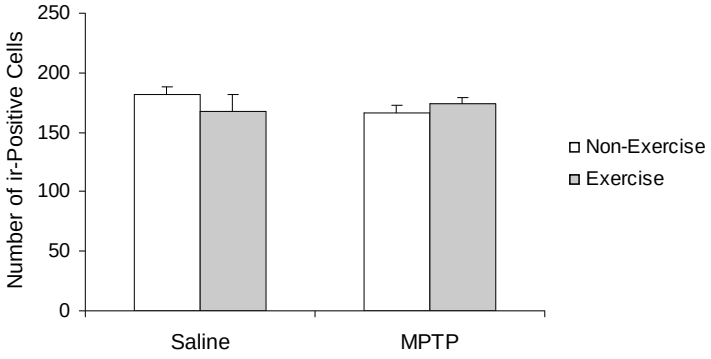



**DARPP-32**

Figure 8: Immunohistochemical staining for the effector molecule dopamine and adenosine cAMP responsive protein phosphatase or 32 kDa in the dorsolateral striatum.




# DARPP-32 Thr75

Figure 9: Immunohistochemical staining for the effector molecule dopamine and adenosine cAMP responsive protein phosphatase or 32 kDa phosphorylated at position Thr 75 in the dorsolateral striatum.

Figure 4: qRT-PCR analysis of the relative expression of GluR1 and its alternative slice variants flip and flop in the dorsolateral striatum in saline and MPTP-lesioned mice subjected to intensive treadmill exercise.

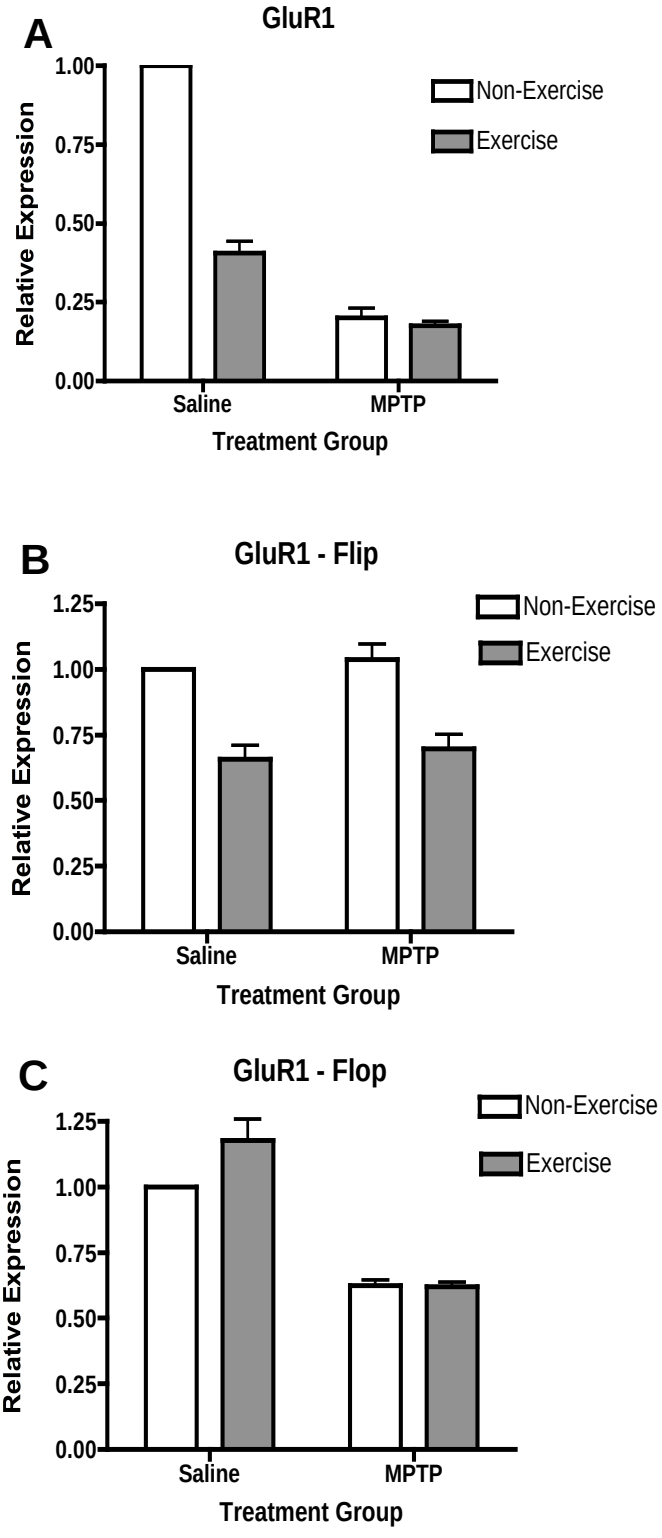


Figure 7: qRT-PCR analysis of the relative expression of GluR2 and its alternative slice variants flip and flop in the dorsolateral striatum in saline and MPTP-lesioned mice subjected to intensive treadmill exercise.

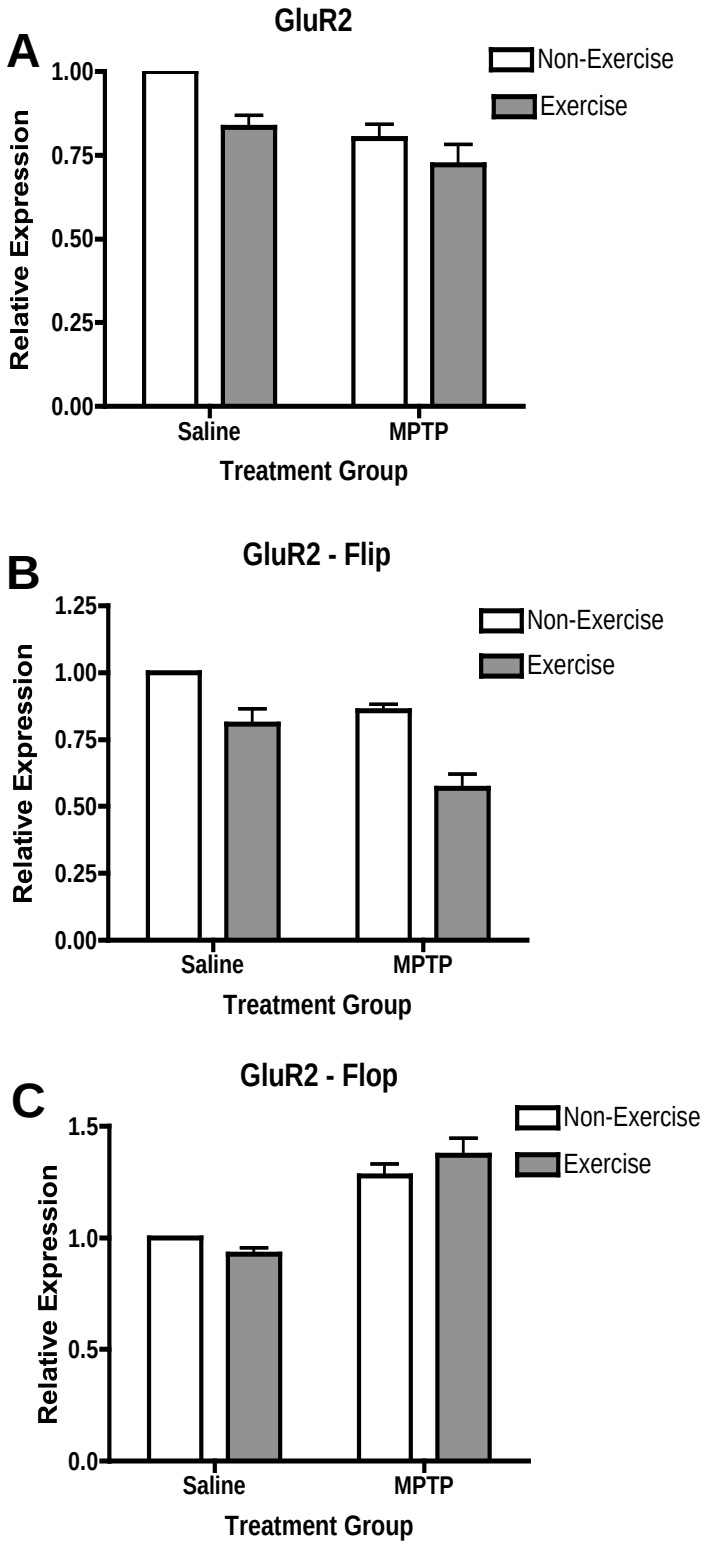


Table 3: Summary of findings for GluR1 and GluR2 transcripts and proteins with intensive treadmill exercise in the mouse.

| Probe | MPTP | Exercise | Interaction |
|---|---|---|---|
| GluR1-pan protein | | | |
| cell counts | ↓ T | - | - |
| neuropil | - | - | - |
| GluR1~Ser845 protein | | | |
| cell counts | - | - | - |
| neuropil | - | - | - |
| soma density | - | - | + 1 |
| GluR1-pan mRNA | ↓ | ↓ | + 2 |
| GluR1-flip mRNA | - | ↓ | - |
| GluR1-flop mRNA | ↓ | - | - |
| GluR2-pan protein | | | |
| cell counts | ↑ | - | + 3T |
| neuropil | - | - | - |
| arbor | ↓ | - | ↑ 4 |
| GluR2~Ser880 protein | | | |
| cell counts | - | ↑ | - |
| neuropil | - | - | - |
| soma density | - | ↓ 5 | |
| GluR2-pan mRNA | ↓ | ↓ | - |
| GluR2-flip mRNA | ↓ | ↓ | - |
| GluR2-flop mRNA | ↑ | - | - |
| DARPP-32 protein | - | ↓ T | - |
| DARPP-32~PhosphoThr$_{75}$ | - | - | - |

T: Denotes trend
1: Increase in MPTP+exercise vs MPTP no exercise; decrease in saline+exercise vs saline no exercise.
2: Decrease saline+exercise vs saline no exercise.
3: Increase in MPTP+exercise vs MPTP no exercise (trend).
4: Increase in saline+exercise vs saline no exercise.
5: Decreased uniform staining in saline group with exercise.

# Reportable Outcomes

**Component 1: To test the hypothesis that exercise enhances neuroplasticity of the MPTP-lesioned mouse through glutamate by modulating dopamine biosynthesis.**

***Study 1****: The level of striatal dopamine and its metabolites will be determined using HPLC analysis comparing exercise versus non-exercise groups in the MPTP-lesioned mouse.*

This Aim has been completed. The findings are described in our recent publication Petzinger et al 2007 in *Journal of Neuroscience*. This manuscript is included in the Appendix of this report. Briefly, with exercise there is no enhanced return of striatal dopamine in the MPTP-lesioned mouse but rather we observed increased release of dopamine in surviving nigrostriatal dopaminergic projections with exercise. This analysis was made using fast-scan cyclic voltammetry.

There are no deviations from Study 1. However, to strengthen and further understand this phenomenon of alterations in dopamine with exercise, we have added two important studies: (i) fast-cyclic voltammetry; and (ii) accelerated mouse rotarod. The fast-scan cyclic voltammetry allowed us to discover that with exercise there is enhanced dopamine release in the MPTP-lesioned mouse model. These findings are described in Petzinger et al 2007, included in the Appendix of this report. The addition of the rotarod analysis of motor behavior allowed us to determine that there is in fact enhanced motor learning in the MPTP-lesioned mouse subjected to intensive treadmill exercise. These findings are also described in Petzinger et al 2007.

***Study 2****: The pattern of expression of striatal tyrosine hydroxylase (TH), dopamine transporter (DAT), cAMP-responsive enhancer binding protein (CREB), phospho~CREB, and dopamine- and adenosine- 3':5'-monophosphate-regulated phosphoprotein (DARPP-32), and phospho~DARPP-32 protein and their mRNA transcripts in surviving dopaminergic neurons will be determined using immunohistochemistry, western immunoblotting, in situ hybridization and correlated with striatal dopamine return. Pilot data shows attenuation of the return of DAT protein, and TH mRNA by exercise in MPTP-lesioned mice*.

This aim is near completion. We have completed the analysis of TH and DAT proteins using western immunoblotting and immunohistochemical staining, and the analysis of their mRNA transcripts using in situ hybridization histochemistry. These findings are described in Fisher et al 2004 and Petzinger et al 2007, both included in the Appendix of this report. The analysis of DARPP-32 and its phosphorylated isoform at Thr75 is described in Petzinger et al 2007. CREB and phospho-CREB are planned for completion in Year 4. The delay is primarily based on technical difficulties with the antibody probes and the verification of their antigen specificity.

There were no deviations from this aim.

***Study 3:*** *The effect of exercise on glutamatergic synapses in the striatum after injury will be determined using ultrastructural immunohistochemical staining with electron microscopy. Pilot data shows altered glutamatergic synapses using immuno-electron microscopy*.

This aim has been completed. Results from this study using immuno-electron microscopy with an antibody against glutamate are presented in the published manuscript Fisher et al 2004 included as an Appendix in this report.

There were no deviations from this aim.

***Study 4:*** *The pattern of expression of subunits for both the NMDA and AMPA receptor subtypes and their phosphorylated state will be determined using western immunoblotting, immunocytochemistry and in situ hybridization histochemistry*.

The first phase of this aim, analyzing the pattern of expression of the AMPA receptor subunits GluR1 and GluR2 using qRT-PCR and immunohistochemical staining within the dorsolateral striatum, has been completed. This analysis also included determination of the pattern of expression of the phosphorylated forms of these protein subunits as well as the expression of the flip and flop mRNA isoforms due to alternative splicing. These data are included in this report and are the basis of a manuscript to be submitted very soon. We have also used qRT-PCR for the analysis of AMPA receptor subunits GluR2 and GluR3 as well as the NMDA receptor subunits NR1, NR2A through NR2D. We anticipate these findings to be included as a manuscript in the near future.

We have deviated from this aim with the addition of Golgi staining to determine potential alterations in the pattern of dendritic spine density. Our initial analysis included quantification of dendritic spine density within the dorsolateral striatum of mice from all four groups including (i) saline control, (ii) saline+exercise, (iii) MPTP-lesioned, and (iv) MPTP+exercise. The rationale for this approach is the fact that dendritic spine density is influenced by glutamatergic neurotransmission. WE do observe MPTP-dependent changes comparing saline and MPTP-lesioned groups. At the writing of this report we have not been able to confirm differences that are exercise-dependent. We suspect that there are differences but their discovery are masked by our inability to delineate between the direct (dopamine receptor D1-dependent) and indirect (dopamine receptor D2-dependent) striatal projection pathways using this approach since Golgi stain occurs in a subset of all neurons. Since we observe changes in dopamine receptor D2 with exercise (as outlined in Fisher et al 2004) we hypothesize that changes in dendritic spine density may be localized to this pathway. Therefore, we have initiated a study utilizing two transgenic strain of mice expressing green fluorescent protein in ether the D1-dependent or D2-dependent striatal projection populations. These strains have just been obtained and are now being bred to generate sufficient numbers for experimentation.

We have deviated from this aim with the addition of quantitative real-time PCR using an Eppendorf realplex thermocycler recently acquired by our lab. This approach will allow for the analysis of mRNA transcripts to complement in situ studies and protein expression in our lesioning and exercise paradigm. Findings from this approach are included in this report and will be part of an upcoming manuscript to be submitted for publication.

***Study 5****: We will test the hypothesis that exercise induced neuroplasticity can be attenuated through the administration of either a NMDA or AMPA receptor antagonist. After MPTP-lesioning mice will be subjected to exercise while receiving either the NMDA receptor antagonist MK-801 or the AMPA receptor antagonist GYKI-52466. Behavioral recovery will be compared between groups. Brain tissue will be analyzed for alteration in dopaminergic function (dopamine, DAT and TH expression). Pilot studies show that both glutamate receptor antagonists GYKI-52466 and MK-801 can be administered in this model of MPTP-lesioning*.

The initial phase of this aim has been completed. We have administered both an AMPA receptor antagonist (GYKI-52466) and a NMDA receptor antagonist (MK-801) to mice administered either saline or MPTP. Findings suggest that the administration of glutamate antagonist attenuates the recovery of the nigrostriatal dopaminergic system following MPTP-lesioning. The next phase is to subject mice to these same glutamate antagonists while they are undergoing intensive treadmill exercise. At this point we intend to deviate from the initial experimental design in our approach but should reach the same scientific conclusions. In collaboration with Dr. John Wash (USC Andrus Gerontology Center) we have pursued the analysis of the glutamatergic contribution to exercise-enhanced neuroplasticity in striatal slice cultures in our model of lesioning and exercise. Some initial findings using fast-scan cyclic voltammetry that show altered dopamine release in MPTP-lesioned mice subjected to intensive treadmill exercise have been included as an important part of our manuscript Petzinger et al 2007 in the Journal of Neuroscience (included in the Appendix). Studies currently underway include the examination of changes in the ration of AMPA to NMDA currents within the dorsolateral striatum, analysis of long-tern potentiation and long-term depression with

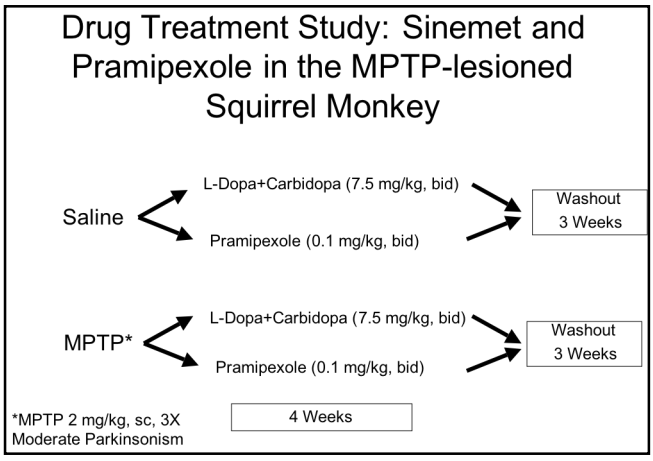
exercise, as well as pharmacological examination of glutamatergic currents using subunit specific antagonists to delineate changes in channel composition in both NMDA and AMPA receptors. Studies examining changes in the AMPA to NMDA receptor currents with exercise are to be included in an upcoming manuscript.

Studies have been carried out using immuno-electron microscopy with an antibody against glutamate showing no significant alteration in glutamate synaptic occupancy in mice administered the AMPA receptor antagonists GYKI-52466 or the NMDA receptor antagonists MK-801.

**Component 2: Pharmacological Enhancement of Neuroplasticity in the MPTP-lesioned Non-Human Primate Model.**

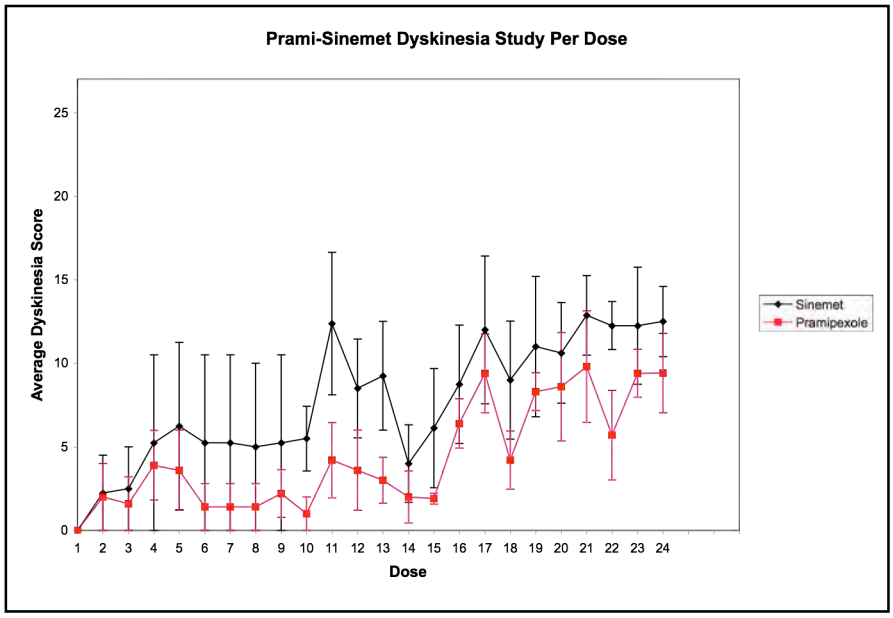
***Study 1:*** *The behavioral recovery of saline injected and MPTP-lesioned squirrel monkeys will be compared with and without the administration of Pramipexole. Animal behavior will be monitored using both a cage side clinical rating scale and a personal activity monitor.*

An additional phase of this aim was to characterize the behavioral, morphological, and neurochemical neuroplasticity (recovery) in the MPTP-lesioned nonhuman primate without pharmacological treatment. The analysis of TH, DAT, midbrain dopaminergic neurons, motor behavior, and levels of CPu dopamine were evaluated in squirrel monkeys administered MPTP in a series of 6 or 2 injections (subcutaneous, 2 mg/kg free-base, 2 weeks between injections) over several months. Six injections corresponded to a moderate parkinsonian state and two injections to a mild parkinsonian state. These findings are described in detail in a recently published manuscript Petzinger et al, 2006, which is included in the appendix of this report. One interesting finding of this study was that the return of normal motor behavior at 9 months in MPTP-lesioned squirrel monkeys rendered moderately parkinsonian, was accompanied by an incomplete return of striatal dopamine. Specifically we observed a greater than 90% level of total dopamine depletion in the dorsolateral putamen at 9 months when animals were fully recovered. While it has been reported that pre-synaptic adaptations in remaining dopaminergic neurons and terminals are thought to lead to normalization of extracellular levels of dopamine especially in animals with lesions resulting in less than 80% depletion, it has also been shown that animals with greater than 80% depletion show only partial normalization of extracellular striatal dopamine (Castaneda 1990). We carried out voltammetry studies in a subset of moderately parkinsonian animals at 9 months post-MPTP lesioning, to examine changes within the nigrostriatal terminals. We observed that the dopaminergic terminals in the dorsolateral striatum remained substantially depleted (> 90%) (see Study 4 below) along with the total levels of striatal dopamine. As other investigators have reported, however, we observed was a greater degree of total dopamine return in the ventral striatum and a normalization of dopamine turnover. Tyrosine hydroxylase and DAT expression was increased in late stage recovery even in dopamine depleted regions and supports a role for sprouting. We also observed an increase in DARPP-32 expression within medium spiny neurons of recovered animals, which supports the role of post-synaptic compensatory changes as an underlying mechanism of this recovery.




The main component of aim 1 was to investigate the long-term effect of dopamine replacement therapy on the behavioral recovery of the MPTP-lesioned squirrel monkey. For study 1 we treated MPTP-nonhuman primates with either Pramipexole or L-DOPA/ Carbidopa for 4 weeks (3 days/week, twice daily, Tues-Thurs) and then washed out for 3 weeks. A baseline evaluation was performed on week 1, animals were treated with dopamine replacement therapy from week 2-5, and then animals were washed out weeks 6-8. We added a Sinemet (L-DOPA/ Carbidopa) group for comparison with Pramipexole based on the scientific

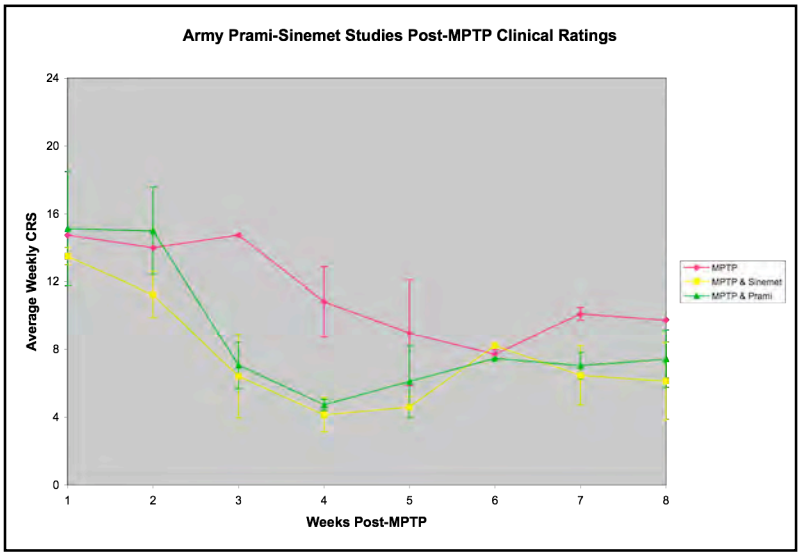
rationale that L-dopa, unlike Pramipexole, is metabolized and stored by dopaminergic terminals and therefore may have a more direct effect on the regulation of endogenous dopamine production and behavioral recovery and offers an interesting comparison to a compound that is not taken up by terminals.




One important outcome in Study 1 was the unexpected induction of dyskinesia in the MPTP-lesioned animals administered Pramipexole. This new finding has not been reported in the literature by other investigators and we are preparing a manuscript reporting this novel finding (Figure 8). This may indicate that Sinemet and Pramipexole may both induce dyskinesias through similar mechanisms.

**Figure 8**

The behavioral assessment in this first group of animals carried out up to 8 weeks after the last injection of MPTP showed a slight enhancement of behavioral recovery in both the Sinemet and Pramipexole groups versus the saline treated group (Figure 9).



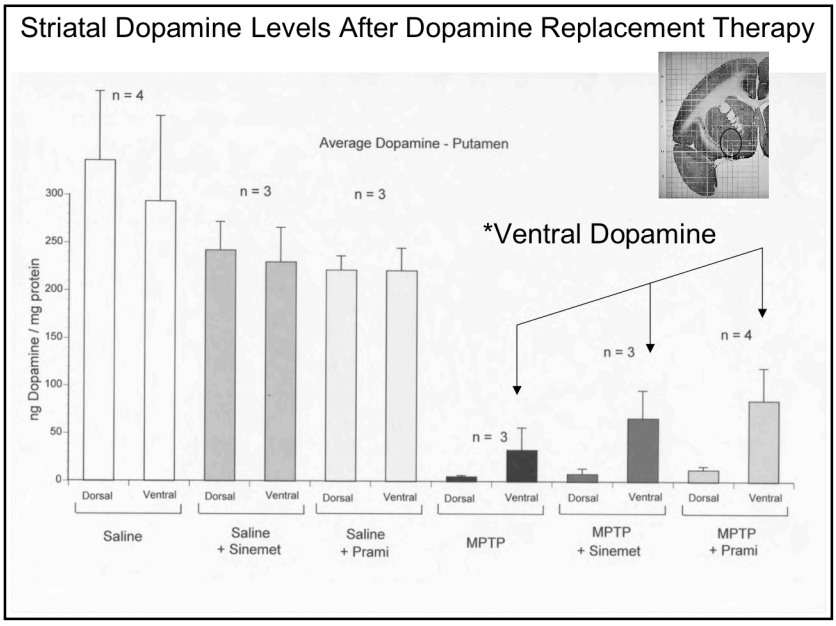

Nonhuman primates that were initially to be used for the long-term behavioral studies, were used for (1) adding a L-dopa group (2) conducting microdialysis studies to extend our findings in Study 2 (neurochemistry); and (3) to examine alterations in electrophysiolgical properties of corticostriatal neurons and voltammetry. See Sections below.

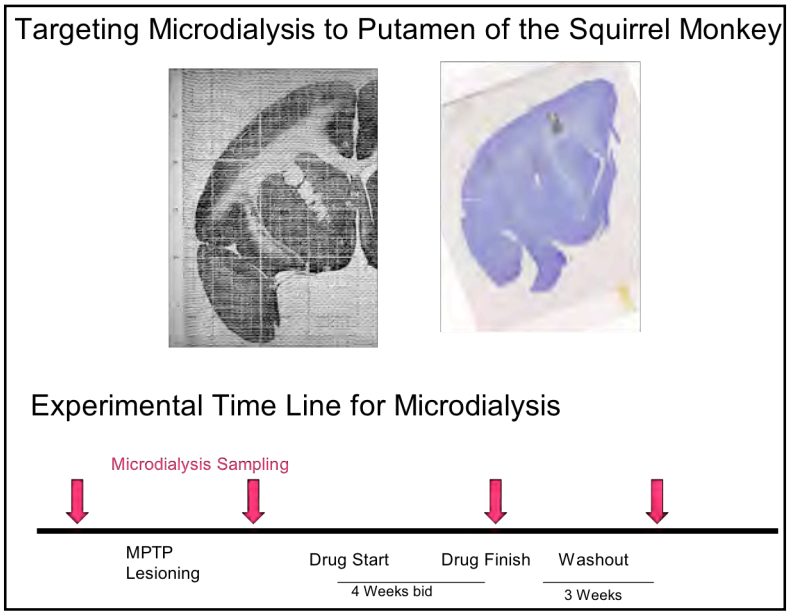
***Study 2**: Analysis of brain tissue from MPTP-lesioned squirrel monkeys administered Pramipexole or L-dopa/carbidopa or saline. This analysis included neurochemistry and molecular studies that examined the pattern of expression of proteins and mRNA transcripts important for dopaminergic function (including TH, DAT, VMAT2) at the level of the SNpc and CPu.*

Studies under this aim included (1) HPLC analysis of dopamine and its metabolites in the dorsal and ventral caudate nucleus and putamen, (2) Microdialysis of dopamine in the putamen, and (3) western immunoblot analysis of CPu proteins including TH, DAT, VMAT-2, DARPP-32, DARPP-32~phosphoThr34, and DARPP-32~phosphoThr75. Immunohistochemical staining for these same proteins are currently underway and their analysis is not yet completed. The following section highlights our current findings using these techniques for Study 2.

(1) Total dopamine levels and metabolites were analyzed from all groups of animals, using HPLC. Brains were removed from all groups at completion of 4 weeks of drug or saline treatment, followed by 3 weeks of drug washout. Microdialysis of the putamen was added to this study to complement and support findings from out HPLC analysis of tissue. We found there was a slight increase in dopamine levels in the ventral caudate and putamen of animals receiving Pramipexole or Sinemet. These results are shown in Figure 10.

**Figure 10:** Analysis of dopamine and its metabolites in the MPTP-lesioned squirrel monkey. Squirrel monkeys were MPTP-lesioned and then treated one week after the last injection of MPTP with either Sinemet (10 mg/kg twice daily), or Pramipexole (1 mg/kg twice daily). Animals were treated for four weeks. On each week animals received drug for three days (Tue, Wed, Thurs)) and then saline for four days (Fri, Sat, Sun, Mon). Animals were rated each day for parkinsonian features and for dyskinesia. Drug was washed out for 3 weeks and then animals were euthanized. Brain tissue was collected and striatal tissue dissected 8 weeks (1 week monitoring + 4 weeks drug treatment + 3 weeks washout) after MPTP. HPLC analysis showed that Pramipexole and Sinemet (L-dopa + carbidopa) treated animals had a slight increase in striatal dopamine, especially in the ventral putamen, compared to MPTP + saline treated nonhuman primates.






(2) Microdialysis was carried out to determine in vivo levels of dopamine within the putamen. Briefly, microdialysis was carried out on 3 squirrel monkeys prior to MPTP-lesioning (as baseline), immediately following MPTP lesioning, during treatment with either pramipexole or Sinemet, and again after a 3 week washout. The primary finding was that pramipexole treated animals displayed elevated amphetamine-evoked dopamine release compared to MPTP untreated animals. Sinemet animals had a level of dopamine release intermediate to these two groups. The adjacent Figure shows the timeline of microdialysis studies. Figure 12 shows HPLC analysis of dopamine levels from microdialysis.

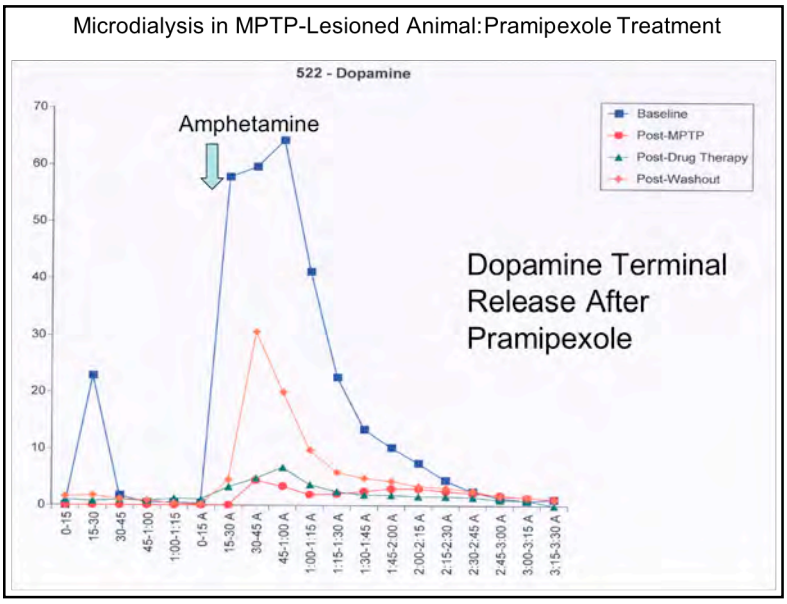
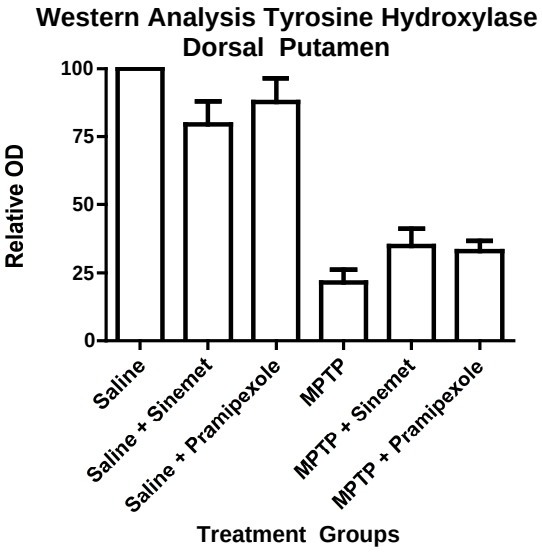



**Figure 12** above shows a representative microdialysis experiment with the same animal used as its own control and undergoing repeated microdialysis studies. Our studies show that Pramipexole or Sinemet treated animals have greater amphetamine-induced dopamine release.

(3) Western immunoblot analysis of proteins for TH, DAT, VMAT-2, and DARPP32 were carried out on tissues derived from either the ventral or dorsal caudate nucleus or ventral or dorsal putamen. Figure 13 displays a subset of the analysis of the western immunoblot data. To summarize, we found that within the dorsal caudate and putamen there is a slight elevation of TH and DAT expression in MPTP-lesioned animals treated with either Sinemet or pramipexole. There was no apparent change in VMAT-2 expression between all lesioned groups. There appears to be elevated DARPP-32 expression in MPTP-lesioned animals treated with Sinemet. There was also an increase in the phosphorylated forms of DARPP-32 (~phosphoThr34, and ~phosphoThr75) in Sinemet treated animals that was not observed in Pramipexole treated animals. We are currently analyzing the remaining western immunoblots and determining if our

findings are consistent amongst the groups. We are also finishing the western blot analysis of the ventral caudate and putamen. These analyses plus the determination of the profiles of expression of the dopamine receptors will be completed in year 4 of this proposal. These studies will be complemented with immunohistochemical staining of tissues sections with antibody probes against TH, DAT, DARPP-32, and VMAT-2. Staining of sections has just been completed and are currently under analysis.

The following pages are Figure 13, which are a representative collection of western immunoblot analysis of TH, DAT, DARPP-32, and VMAT-2. Several figures without error bars are the analysis of a single western immuoblot gel. Figures with error bars represent analysis of several immunoblot gels. Studies are underway to evaluate the pattern of expression of these proteins in the dorsal and ventral regions of the caudate nucleus and putamen.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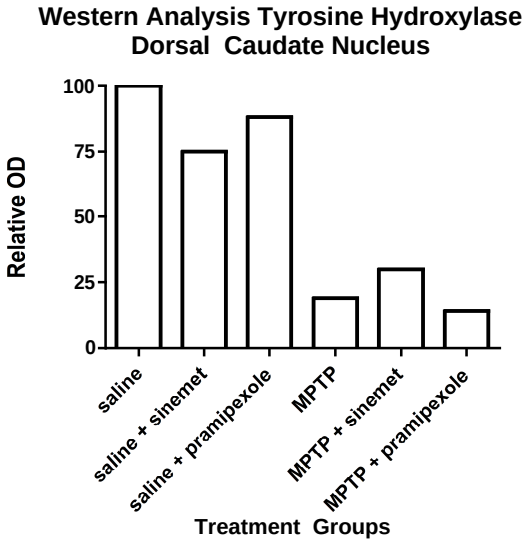
Dorsal Putamen
Relative OD
100
75
50
25
0
Saline
Saline + Sinemet
Saline + Pramipexole
MPTP
MPTP + Sinemet
MPTP + Pramipexole
Treatment Groups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Dorsal Caudate Nucleus
Relative OD
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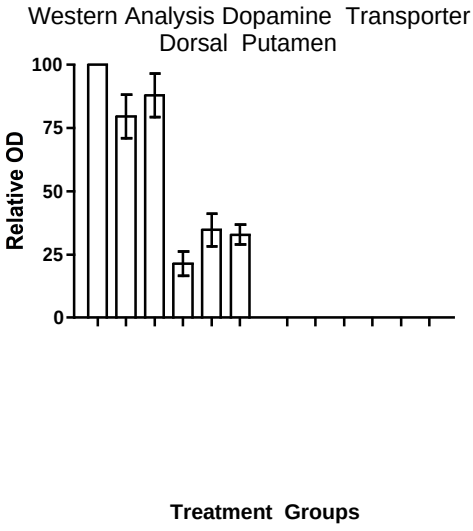
saline + sinemet
saline + pramipexole
MPTP
MPTP + sinemet
MPTP + pramipexole
Treatment Groups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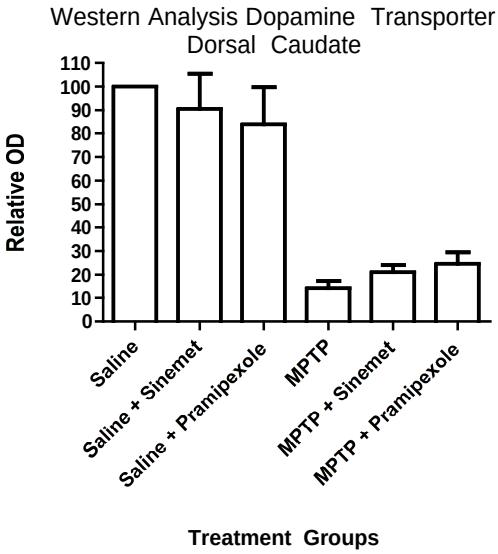
Dorsal Putamen
Relative OD
100
75
50
25
0
Treatment Groups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Dorsal Caudate
Relative OD
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70
60
50
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30
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10
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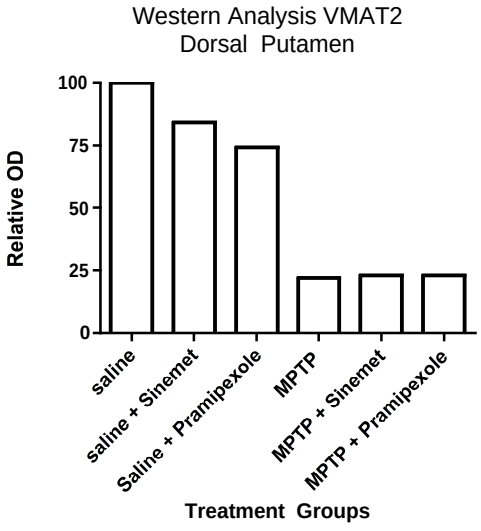
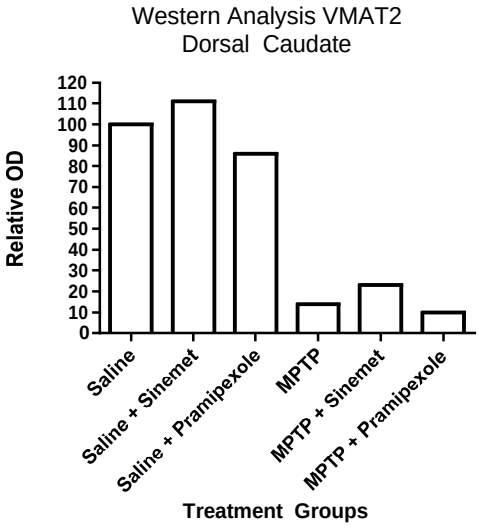
Saline + Pramipexole
MPTP
MPTP + Sinemet
MPTP + Pramipexole
Treatment Groups


Western Analysis VMAT2
Dorsal Putamen
Relative OD
100
75
50
25
0
saline
saline + Sinemet
Saline + Pramipexole
MPTP
MPTP + Sinemet
MPTP + Pramipexole
Treatment Groups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Dorsal Caudate
Relative OD
120
110
100
90
80
70
60
50
40
30
20
10
0
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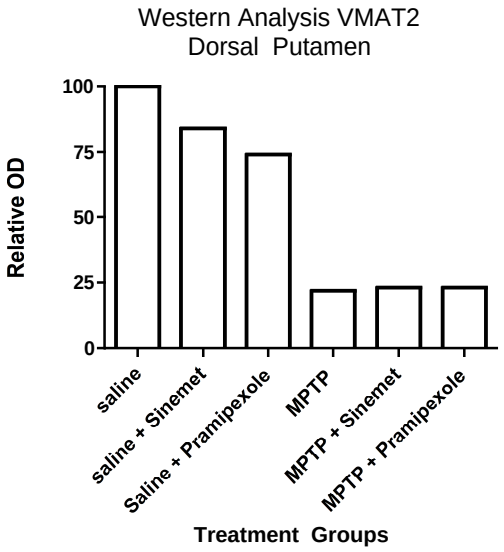
Saline + Sinemet
Saline + Pramipexole
MPTP
MPTP + Sinemet
MPTP + Pramipexole
Treatment Groups


Western Analysis VMAT2
Dorsal Putamen
Relative OD
100
75
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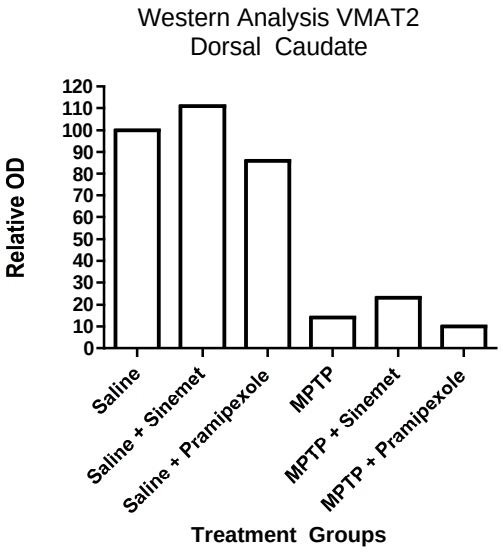
saline + Sinemet
Saline + Pramipexole
MPTP
MPTP + Sinemet
MPTP + Pramipexole
Treatment Groups


Western Analysis VMAT2
Dorsal Caudate
Relative OD
120
110
100
90
80
70
60
50
40
30
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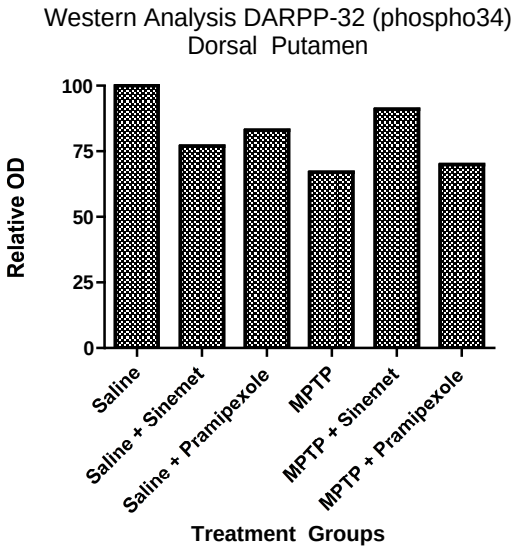
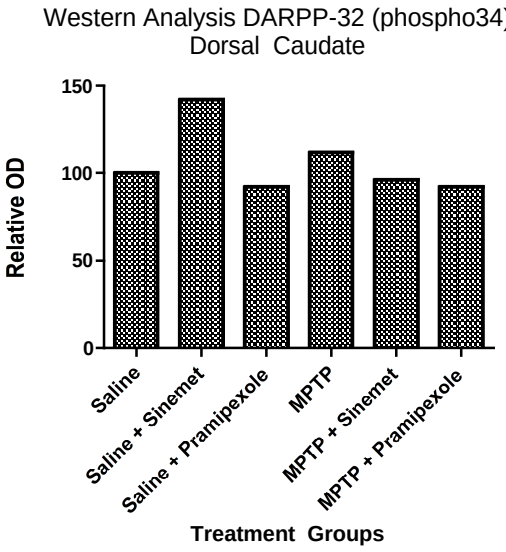
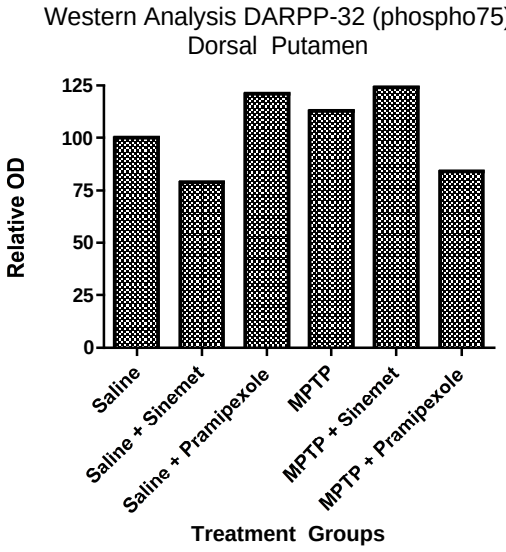
Saline + Sinemet
Saline + Pramipexole
MPTP
MPTP + Sinemet
MPTP + Pramipexole
Treatment Groups


Western Analysis DARPP-32 (phospho34)
Dorsal Putamen
Relative OD
100
75
50
25
0
Saline
Saline + Sinemet
Saline + Pramipexole
MPTP
MPTP + Sinemet
MPTP + Pramipexole
Treatment Groups


Western Analysis DARPP-32 (phospho34)
Dorsal Caudate
Relative OD
150
100
50
0
Saline
Saline + Sinemet
Saline + Pramipexole
MPTP
MPTP + Sinemet
MPTP + Pramipexole
Treatment Groups

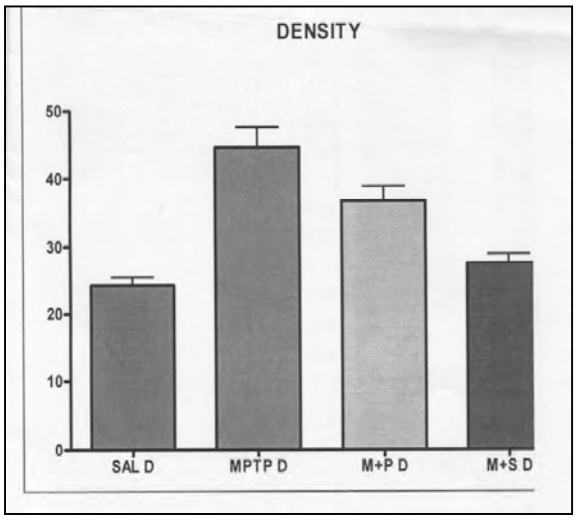

Western Analysis DARPP-32 (phospho75)
Dorsal Putamen
Relative OD
125
100
75
50
25
0
Saline
Saline + Sinemet
Saline + Pramipexole
MPTP
MPTP + Sinemet
MPTP + Pramipexole
Treatment Groups

***Study 3:*** *The pattern of expression of the dopamine receptors D1, D2, and D3 will be determined in both the SNpc and CPu. The level of protein expression will be determined western immunoblotting, immunohistochemistry, while the level of mRNA transcript expression will be determined using in situ hybridization histochemistry. Double labeling techniques will be used to co-localize the dopamine receptor changes with other enkephalin or substance P containing neurons. Preliminary data supports our ability to use these techniques in the non-human primate*.

This aim will be carried out in year 4. We have designed primer sets for analyses using qRT-PCR and have evaluated a number of different antibody probes against dopamine receptors D1, D2, and D3 for use in both western immunoblotting and immunohistochemical staining.

***Study 4:*** *The effect of Pramipexole on glutamatergic synapses in the striatum after injury will be determined using ultrastructural immunohistochemical staining with electron microscopy. Pilot data shows our ability to quantify glutamatergic synapses using immuno-electron microscopy.*

In collaboration with Dr. Charles Meshul (Oregon Health Sciences University, Portland, OR) perfusion fixed brain tissues were harvested from a nonhuman primate from each group for analysis using immuno-electron microscopy with an antibody against glutamate. These results are summarized in the Figure below. Following MPTP-lesioning there is an increase in the relative density of striatal glutamate immunolabeling (second bar) within corticostriatal terminals. After treating MPTP-lesioned animals with Pramipexole or Sinemet the relative density of glutamate immunolabeling is reduced. Increased density of striatal glutamate within the terminal is thought to reflect decreased glutamate release. Our study would suggest that Sinemet increases glutamate release to a slightly greater extent than Pramipexole. This increased glutamate release may be one means by which dyskinesia is elicited to a greater extent in Sinemet treated animals than Pramipexole treated animals.




The following studies have been added to this proposal with the aim of understanding the impact of alterations in the expression of glutamate receptors on the electrophysiological properties of striatal neurons.

**Electrophysiological Studies of Basal Ganglia Function the Nonhuman Primate Model of PD**

An important aspect of the nonhuman primate is the anatomical similarity of basal ganglia structure and function to that of humans, thereby providing an important tool for investigating basal ganglia function, such as neurophysiological properties in the normal and disease state, and thus serves as the foundation for identifying new therapeutic treatments. For example neurophysiological studies have implicated over-activity at corticostriatal synapses as one underlying mechanism for the development of motor impairment in PD (Konitsiotis *et al.*, 2000; Soares *et al.*, 2004; Wichmann and DeLong, 2003). Electrophysiological studies in our labs, using the MPTP-lesioned squirrel monkey, have shown changes in AMPA and GABA mediated synaptic neurotransmission that may account for excessive excitatory corticostriatal drive. For these studies, we administered MPTP in a series of 6 subcutaneous injections of 2.0 mg/kg (free-base) every 2 weeks for a total of 12 mg/kg. Whole brains were harvested at either 6 weeks (when animals are parkinsonian) or 9 months (when animals are motorically recovered) after the last injection of MPTP and striatal synaptic function was examined in coronal *in vitro* brain slices. We found that the input/output relationship was greater for AMPA receptor mediated synaptic currents at 6 weeks after MPTP-lesioning compared to saline control using whole cell voltage clamp. The relative strength of $GABA_A$ versus AMPA receptor mediated synaptic responses was calculated as the $I_{GABA-}$ changes in $_A$ / $I_{AMPA}$ ratio. Interestingly,

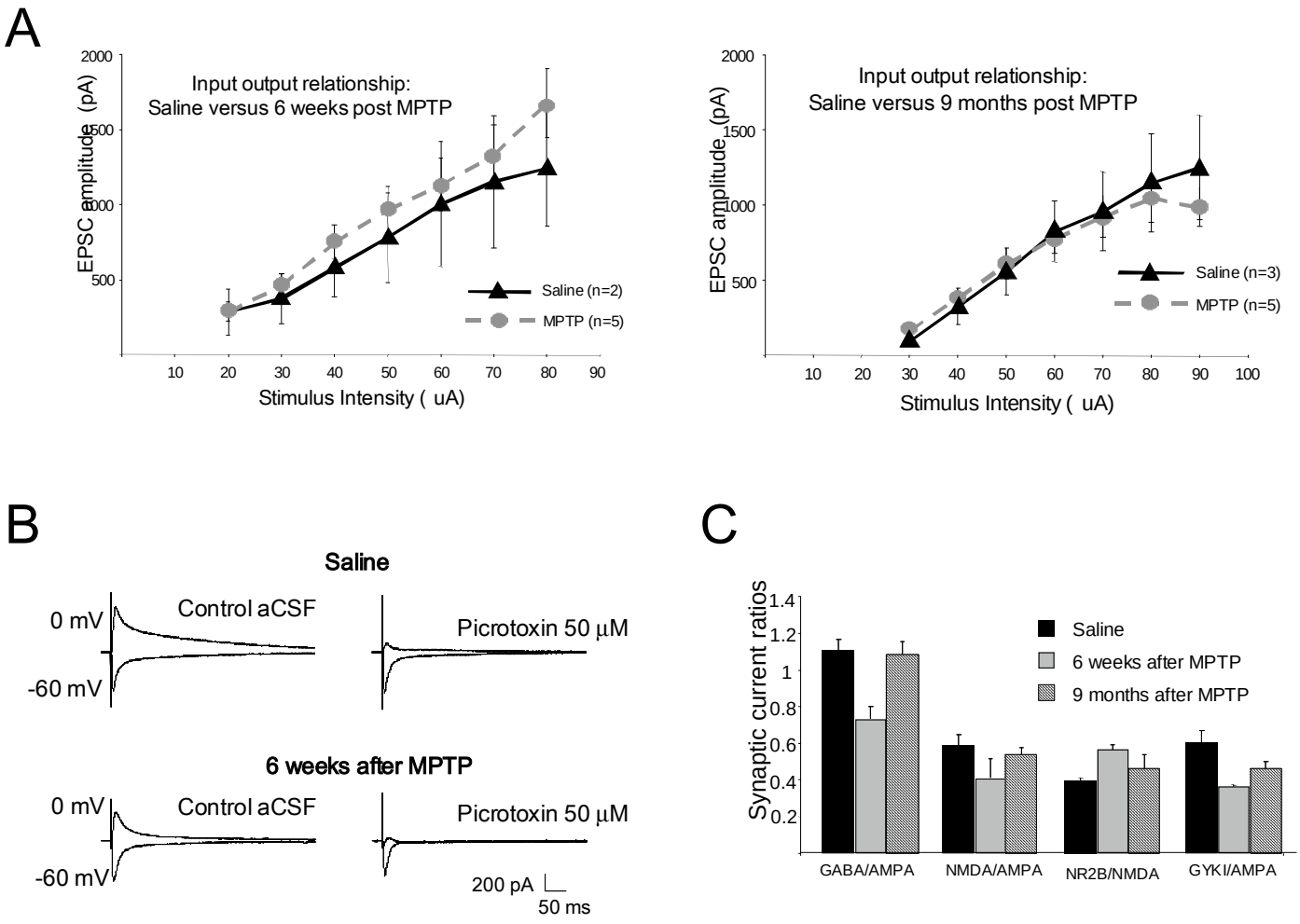
we also found a reduced $I_{GABA\text{-}A}/I_{AMPA}$ ratio 6 weeks after MPTP. These GABAergic inhibition that we and others have observed may play an important role in facilitating the synchrony and oscillatory patterns of discharge found throughout the basal ganglia motor circuit in MPTP-treated akinetic primates (Goldberg *et al.*, 2002; Raz *et al.*, 1996; Raz *et al.*, 2001). Analysis of animals 9 months after MPTP administration suggests there is normalization of corticostriatal hyperactivity when animals demonstrate full behavioral recovery. Specifically we found the input/output ratio for AMPA receptor-mediated synaptic responses and the $I_{GABA\text{-}A}$ / $I_{AMPA}$ ratio returned back to control levels (Figure 3). These observations are in agreement with the view that excessive glutamatergic corticostriatal synaptic function may be a contributing factor to the behavioral pathology of PD (Konitsiotis *et al.*, 2000; Muriel *et al.*, 2001). Future studies will exam whether changes in glutamatergic drive in fully recovered animals differentially impacts corticostriatal synapses in direct versus indirect basal ganglia pathways, as has been reported in the parkinsonian state (Day *et al.*, 2006; Wichmann and DeLong, 2003).

Dopamine denervation in animal models of PD is also associated with changes in the molecular composition of AMPA and NMDA receptors in the striatum (Betarbet *et al.*, 2004; Betarbet *et al.*, 2000; Hallett *et al.*, 2005; Hurley *et al.*, 2005; Nash *et al.*, 2004). We also found evidence for changes in the pharmacological profile of AMPA and NMDA receptors, which are consistent with these molecular studies. For example as shown in Figure 3, in animals examined 6 weeks post MPTP-lesioning, we found; (i) a decrease in the $I_{NMDA}/I_{AMPA}$ ratio; (ii) an alteration in the NMDA receptor subunit composition as indicated by increased sensitivity to the selective NR2B antagonist CP-101,606; and (iii) an alteration in AMPA receptor mediated synaptic responses, as indicated by changes in the sensitivity to the selective AMPA receptor antagonist, GYKI-52466 compared to saline control animals (Nash *et al*., 2004; Ruel *et al.*, 2002). Again, with behavioral recovery at 9 months post-MPTP-lesioning, we observed the trend of a return of NMDA and AMPA receptor function to match that seen in saline injected squirrel monkeys (Figure 14).

The glutamatergic corticostriatal and the dopaminergic nigrostriatal system are important mediators of synaptic plasticity, termed long-term depression (LTD) and long-term potentiation (LTP), within the basal ganglia (Centonze *et al.*, 2001; Mahon *et al.*, 2004; Picconi *et al.*, 2005; Reynolds and Wickens, 2002). Electrophysiological studies in our lab, using saline control squirrel monkeys, have shown that the induction of long-term synaptic plasticity at corticostriatal synapses is region specific, with LTP being induced in more medial regions and LTD in more lateral regions. These findings agree with previous reports from the rodent model of PD (Partridge *et al.*, 2000; Smith *et al.*, 2001). Studies in the rat have shown a loss of synaptic plasticity after 6-OHDA administration, which we have observed in the MPTP-lesioned mouse model, 1 to 2 weeks after neurotoxicant exposure (Calabresi *et al.*, 1992; Centonze *et al.*, 1999; Kreitzer and Malenka, 2007). Presently, there is little known regarding alterations in synaptic plasticity immediately following MPTP-lesioning in the nonhuman primate.

Analysis of the expression of synaptic plasticity in the squirrel monkey 9 months after MPTP-lesioning has shown that LTD and LTP expression is evident. In the same animals used for analysis of glutamate neurotransmission above, we observed a dramatic and permanent decrease in dopamine release as measured by fast-scan cyclic voltammetry (Cragg, 2003) (Figure 4). This finding is in agreement with previous reports examining dopamine function in the squirrel monkey using HPLC (Petzinger *et al*., 2006). The expression of dopamine-dependent forms of LTP we observed in the dopamine depleted squirrel monkey suggest an adaptation may occur in the expression and/or sensitivity of both D1 and D2 receptors (Centonze *et al*., 2001; Mahon *et al*., 2004; Picconi *et al.*, 2005; Reynolds and Wickens, 2002). Preliminary studies in our lab have shown that LTD expression at lateral cortico-putamen synapses from the 9-month MPTP-lesioned squirrel monkey is D2 dependent, since this effect is blocked by the D2 receptor antagonist *l*-sulpiride. In addition, use of *l*-sulpiride results in the unexpected expression of LTP in lateral synapses (Figure 4). Our findings are consistent with the literature, where dopamine receptors D1 and D2 have been shown to play an important role in LTP and LTD, respectively (Calabresi *et al.,* 1992; Centonze *et al.*, 1999; Wang *et al.,* 2006). Taken together, these data suggest behavioral recovery from MPTP exposure in the squirrel monkey may be due at least in part to compensatory increases in the sensitivity of dopamine receptors, which enables the normal and expected expression of long-term plasticity at corticostriatal synapses.

**Figure 14**: (A) left: In put-output relationship of saline vs MPTP at 6 weeks post-MPTP lesioning. (A) right: Input-output relationship of saline and MPTP-lesioned 9 months after lesioning. (B) and (C) synaptic current ratios for GANA, AMPA, and NMDA in saline, 6-weeks after MPTP and 9-months after MPTP.



# Reportable Outcomes For Years One to Three

## Abstracts:

(1) Society for Neuroscience Annual Meeting, San Diego, 2004 ABSTRACT #1

Behavioral recovery in the MPTP-lesioned nonhuman primate: Altered dopamine biosynthesis and storage.

Hogg, E, M. W. Jakowec, K. L. Nixon, A. T. Abernathy, P. Arevalo, B. E. Fisher, M. Liker, and G. M. Petzinger.

(2) Society for Neuroscience Annual Meeting, Atlanta 2006 ABSTRACT #1

Exercise induced behavioral recovery and plasticity in the MPTP-mouse model of Parkinson's disease.

Jakowec, M. W., P. Arevalo, M. Vuckovic, P. Turnquist, E. Hogg, J. Walsh[#], G. Akopian[#], C. Meshul*, A. Abernathy, M. Ramirez, B. Fisher and G. M. Petzinger.

Dept. Neurology; Davis School of Gerontology[#]; Dept. Biokinesiology and Physical Therapy; University of Southern California, Los Angeles, CA.VA Medical Center*, OHSU, Portland, OR.

The adult brain possesses a tremendous capacity for activity-dependent neuroplasticity. Following injury to the brain, physical therapy plays an important role in promoting recovery. In neurodegenerative disorders such as Parkinson's disease, physical activity improves motor function and may lead to alterations in disease progression. To better understand the role of activity-dependent plasticity in brain repair we are investigating the application of intensive treadmill exercise training in the MPTP mouse model of basal ganglia injury and dopamine depletion. Mice were administered MPTP (4 20 mg/kg each) and subjected to intensive treadmill running for 30 days starting 4 days after the last injection of MPTP (when cell death is complete) at a speed up to 20 meters/minute for 1 hour. During the exercise paradigm, mice were investigated for improvement in behavioral motor features and learning. Harvested brain tissues were analyzed by HPLC for levels of dopamine and its metabolites, and glutamate and the pattern of expression of genes and proteins for tyrosine hydroxylase, dopamine transporter, dopamine receptors D1 and D2, and AMPA and NMDA glutamate receptors using western immunoblotting, immuno histochemistry, and in situ hybridization histochemistry. Electrophysiological analysis of dopamine release was determined using fast cyclic voltammetry on brain slices. Our findings indicated that there was an enhancement of both motor behavior recovery and rotarod learning in exercised mice despite no change in the number of SNpc dopaminergic neurons and the striatal levels of dopamine. Molecular analysis showed down-regulation of DAT and TH, and significant changes in the pattern of expression of ionotropic glutamate receptors in the cortex and striatum. In addition, exercise resulted in an increase in dopamine release compared to MPTP-lesioned mice without exercise. These findings demonstrate that intensive exercise can induce dramatic neuroplasticity in an animal model of basal ganglia injury and provides a valuable framework for supporting exercise in patients with Parkinson's disease.
Supported by grants to J. Walsh (RO1 AG21937), M. Jakowec (RO1 NS44327) and G. Petzinger (US Army NETRP W81XWH-04-1-0444).

(3) Society for Neuroscience Annual Meeting, Atlanta 2006 ABSTRACT #2

Changes in dopamine and glutamate electrophysiology in the MPTP-treated non-human primate and the exercised MPTP-treated mouse. J Walsh*, G Akopian*, M, Jakowec[§], G, Petzinger[§]. USC Neuroscience Program, USC Davis School of Gerontology*, Department of Neurology - USC Keck School of Medicine[§].

We tested the hypotheses that dopamine (DA) and glutamate physiology are altered in the MPTP-treated squirrel monkey using electrophysiological methods. Fast cyclic voltammetry analysis of the monkey putamen revealed that MPTP treatment (6 weeks earlier) resulted in a dramatic loss in DA released in response to intra-putamen stimulation (bipolar tungsten wire electrode, 0.1 msec 100-50 µA stimulus). Saline injected monkeys showed greater DA release in the lateral versus medial putamen. To determine if excitatory amino acid receptor-mediated physiology is altered in the MPTP-treated monkey putamen we applied whole cell voltage clamp techniques and examined the relative contribution of AMPA and NMDA receptors to corticostriatal synaptic events. Saline injected monkeys showed a relatively uniform NMDA/AMPA receptor ratio, while data from MPTP-treated monkeys suggested that two new populations emerged; one with a reduced NMDA/AMPA ratio and another with an enhanced NMDA/AMPA ratio.

We applied a similar strategy to examine the impact of MPTP toxicity on DA and glutamate physiology in the mouse and, more importantly, to determine if changes striatal DA or glutamate physiology tracked the behavioral recovery induced by exercise in the MPTP-treated mouse. Cyclic voltammetry revealed a dramatic reduction in evoked DA release in the striatum of mice treated a month earlier with MPTP. A parallel group of mice were treated with MPTP and exercised daily on a treadmill. MPTP treated mice were significantly compromised in treadmill performance initially but achieved the same performance as saline injected mice by the end of one month of training. The exercise-mediated enhancement of motor skills transferred to a rotarod task. Prior work demonstrated exercise-induced suppression in striatal DAT immunocytochemistry in the MPTP-treated mouse (Fisher et al, 2004, J Neur Res 77:378), but voltammetry revealed a significant exercise-induced increase in DA release in the MPTP treated mouse.

These data demonstrate emergent dopaminergic and glutamatergic plasticity created in the striatum following exposure to the neurotoxin MPTP. We hypothesize these forms of synaptic plasticity underlie both behavioral deficits created early as well as recovery seen later in the MPTP model.

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(4) Society for Neuroscience Annual Meeting, San Diego, CA, 2007. ABSTRACT #1

Dopamine treatment effects on neuroplasticity in the MPTP-lesioned Squirrel Monkey (*Saimiri sciureus*).

G. M. Petzinger*, P. Arevalo*, C. Meshul, E. Hogg*, G. Akopian[#], J. P. Walsh[#], J. VanLeeuwen*, M. Ramirez*, and M. W. Jakowec*.
Department of Neurology*, and Andrus Gerontology Center[#], University of Southern California, Los Angeles, CA and Oregon Health Sciences University, Department of Behavioral Sciences, Portland, OR.

The administration of the neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to the Squirrel monkey (*Saimiri sciureus*) leads to the onset of parkinsonian symptoms due to the loss of nigrostriatal dopaminergic neurons and the depletion of striatal dopamine.

Animals were administered a series of 3 s.c. injections of MPTP (2.0 mg/kg, free-base, 2 wks between injections) or saline as control. Starting 4 wks after the last injection of MPTP, when the time course of cell death is complete, saline and MPTP-lesioned animals were administered either saline, levodopa plus carbidopa (7.5 mg/kg), or the dopamine agonist pramipexole (0.1 mg/kg) 5 days per wk for 4 wks followed by a 3 wks washout. A subset of animals underwent a series of microdialysis studies in conjunction with amphetamine challenge at pre-MPTP-lesioning, post-lesioning but before dopamine treatment, and 4 weeks after completion of dopamine therapy. During drug treatment all animals were subjected to a clinical rating scale to evaluate parkinsonian motor features. At the completion of the washout period brain tissues was collected from all animals and used for analysis of striatal dopamine levels using HPLC, perfusion fixed for immuno-EM, slice culture for electrophysiological studies, and proteins of interest including tyrosine hydroxylase (TH), dopamine transporter (DAT), vesicular monoamine transporter-2 (VMAT-2), and the effector molecule DARPP-32 using western immunoblot and immunohistochemical staining focusing on the caudate nucleus and putamen.

Our findings show that MPTP-lesioned animals treated with Pramipexole or Sinemet showed (1) enhanced amphetamine evoked dopamine release; (2) normalization of Corticostriatal drive; (3) normalization of corticostriatal terminal glutamate density; (4) increased protein expression of TH, DAT, and VMAT-2 and (5) an increase in the phosphorylated forms of DARPP-32.

Our data indicate that in addition to symptomatic treatment of parkinsonian motor features, both dopamine replacement therapy in the form of levodopa or dopamine agonists may lead to enhanced neuroplasticity in the MPTP-lesioned basal ganglia as indicated by the up-regulation of proteins important for dopamine biosynthesis, storage and transmission. The precise mechanism is currently unknown but may involve either direct neurotrophic benefit via dopamine receptor stimulation or enhanced engagement of animals with their environment due to dopamine replacement therapy. This finding raises the issue that starting dopamine replacement therapy early in the course of Parkinson's disease may have additional benefit.

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(5) Society for Neuroscience Annual Meeting, San Diego, CA, 2007. ABSTRACT #2

The role of brain-derived neurotrophic factor (BDNF) over-expression in basal ganglia function and response to exercise in the MPTP-lesioned mouse model.

M. W. Jakowec[§], J. P. Walsh*, G. Akopian*, Pablo Arevalo[§], M. Vuckovic, and G. M. Petzinger[§].
USC Neuroscience Program, USC Davis School of Gerontology*, Department of Neurology, Keck School of Medicine[§], University of Southern California, Los Angeles, CA.

Our previous work has shown that intensive treadmill exercise leads to improved motor performance in the MPTP-lesioned mouse model of basal ganglia injury. In addition, we have observed alterations in components of both the dopaminergic and glutamatergic neurotransmitter systems including altered patterns of expression of genes and proteins encoding receptor subunits as well as increased dopamine release with exercise in MPTP-lesioned mice subjected to intensive treadmill exercise. While the precise link between glutamate and dopamine neurotransmission with exercise is currently unknown, based on reports in the literature, we hypothesize that the neurotrophic factor BDNF may play a role. This factor is central to a number of important aspects of basal ganglia function including synaptogenesis, plasticity, and response to MPTP exposure. For these studies we utilized a transgenic mouse that over-expresses BDNF throughout the brain. BDNF-tg or C57BL/6 mice were administered MPTP in a series of 4 injections (20 mg/kg, i.p., 2 hours apart). A subset of mice was harvested 7 days post-lesioning for analysis of the degree of lesioning by examining protein expression of tyrosine hydroxylase and counting nigrostriatal dopaminergic neurons. Another subset of both BDNF-tg and C57 BL/6 mice were assigned to different groups including (1) saline injected, (2) saline + exercise, (3) MPTP injected, and (4) MPTP + exercise. The exercise regimen was initiated 5 days after the last injection of MPTP, when cell death is completed, and continued for 28 days (5 days/wk) achieving a rate of approximately 20 m/min for 60 minutes each session. Fast-scan cyclic voltammetry (FSCV) was used to examine electrically evoked dopamine release in striatal coronal brain slices. Using this method, dopamine release was sampled in five anatomically distinct sites that varied in dorsal to ventral and medial to lateral dimensions. Analysis of evoked dopamine release showed no release in non-Tg lesioned mice. However, BDNF-tg mice showed small amounts of dopamine release indicating a potential protective role by BDNF. We also examined long-term plasticity at corticostriatal synapses. Control mice demonstrated LTP in medial and LTD at lateral corticostriatal synapses. However, post-MPTP lesioning neither LTP nor LTD could not be evoked as has been reported for the 6-OHDA treated rat (Calabresi et al, 1992; Kreitzer and Malenka, 2007). Our data suggests BDNF over-expression accelerates the recovery of dopamine release and normal expression of long-term plasticity at corticostriatal synapses following exposure to MPTP.

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(6) Society for Neuroscience Annual Meeting, San Diego, CA, 2007 ABSTRACT #3

Altered AMPA receptor expression with exercise in the MPTP-lesioned mouse model of Parkinson's disease.

J. VanLeeuwen*, G. M. Petzinger*, P. Arevalo*, E. Hogg*, G. Akopian#, J. P. Walsh#, M. Ramirez*, and M. W. Jakowec*.
Department of Neurology*, and Andrus Gerontology Center#, University of Southern California, Los Angeles, CA.

We have previously demonstrated that intensive treadmill running leads to motor improvement in the 1-methyl-4-phenyl-1,2,3,6-tetrahyropyridine (MPTP)-lesioned mouse model of Parkinson's Disease. In this study, we investigated changes in the pattern of expression of glutamate receptors and postsynaptic effector molecules to elucidate the molecular modifications that influence the improvement seen in MPTP-lesioned mice after intensive exercise. Four groups of animals were used to examine these changes: (i) Saline; (ii) Saline + Ex; (iii) MPTP; (iv) MPTP + Ex. C57 BL/6 mice were administered four i.p. injections of MPTP (20mg/kg free-base, 2 hours apart) which yields 90% dopamine depletion in the striatum. Exercise was started 5 days (a time point when cell death is complete) after MPTP lesioning and continued for 28 days (5 days a week) using a motorized treadmill. At completion of the exercise regimen, tissue was harvested and the expression of mRNA transcript and protein for the α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) subtype of the glutamate receptor superfamily, as well as the effector molecule DARPP-32, was analyzed within the dorsolateral striatum. The changes in mRNA transcript expression of AMPA receptor subunits, including their alternative splice isoforms, flip and flop, were determined using qRT-PCR analysis. Using immunohistochemical staining, we examined the expression of AMPA receptor subunits, including their phosphorylated states, and the effector molecule DARPP-32. Our results indicate that exercise causes a downregulation of mRNA transcript in the pan forms of GluR1 and GluR2, and in the Flip isoform of the GluR2 subunit. Mice lesioned with MPTP also displayed decreased mRNA for GluR1 and GluR2. Immunostaining revealed changes not accounted for by transcript expression. No significant changes occurred in the expression of the GluR1 protein. We found an upregulation of the GluR2 protein subunit and its phosphorylated state (serine 880) in MPTP + Ex mice. The expression of the effector molecule DARPP-32, was downregulated in exercised mice. These studies showed that exercise influences the pattern of expression of the AMPA receptors within the striatum but that this phenomenon is not explained by mRNA transcript expression, suggesting that alternative mechanisms are involved in this process, such as protein interactions or localization in relation to the synapse. Findings from this study indicate that changes in AMPA receptor subunits may play a key role in putative molecular adaptations that are necessary for activity dependent synaptic plasticity in the dopamine depleted striatum, as is found in the Parkinsonian state.

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(7) Society for Neuroscience Annual Meeting, San Diego, CA, 2007 ABSTRACT #4

Memory impairment and affective behavior in the MPTP-lesioned mouse model of basal ganglia injury.

*M. Vuckovic[1,2], R. I. Wood[2,3], D. P. Holschneider[1,2,3,4,5], D. M. Togasaki[1], G. M. Petzinger[1], M. W. Jakowec[1,2];
[1]Neurol., [2]Neurosci. Grad. Program, [3]Cell and Neurobiology, [4]Psychiatry and Behavioral Sci., [5]Biomed. Engin., USC, Los Angeles, CA.

*Abstract:* Depression, anxiety and dementia are common in patients with Parkinson's disease (PD). Molecular mechanisms connecting the loss of dopamine (DA) with mood and memory disorders are not well understood. The present study investigated whether the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced lesion of basal ganglia in mice can be used as an animal model of memory and affective dysfunction associated with PD. Established mouse behavior tests were used to compare control and MPTP-lesion mice. Depression was measured by tail suspension and sucrose preference. Anxiety was tested using light-dark preference and hole board, and fear was assessed with fear conditioning. Olfactory memory was tested by social transmission of food preference. Separate groups of adult male C57BL/6 mice were evaluated 7 and 30 days after MPTP lesion. Lesioning consisted of 4 i.p. injections of 20 mg/kg MPTP at 2 h intervals. This regimen has been shown to produce severe DA loss in the striatum (up to 90% loss) and 50-70% cell loss in the substantia nigra pars compacta. Control mice received 4 i.p. injections of saline. In the social transmission of food preference test, mice acquired information about novel flavor from a conspecific demonstrator. Subsequently, when presented with two unfamiliar flavors, control mice showed a strong preference (79.0 ± 3.3%) for the flavor consumed by the demonstrator. This preference was significantly decreased in mice 30 days after MPTP (58.7 ± 6.3%, $p < 0.05$), but not 7 days post-lesion (79.1 ± 3.3%). Fear conditioning at 7 and 30 days post-MPTP showed faster extinction of the freezing response to a tone compared to control mice. After 6 minutes of continuous tone exposure, control mice spent significantly more time freezing (43.8 ± 6.0%, $p<0.05$) compared to mice at 7 days (9.6 ± 3.2%) or 30 days post-MPTP (16.5 ± 7.3%). The tail suspension test showed a significant increase in percent of time spent in immobility 30 days compared to 7 days post-lesion (44.2 ± 3.2%, 29.3 ± 3.3%, $p<0.005$), but there was no difference between these two groups compared to control mice (36.6 ± 3.1%). There was no change in sucrose consumption between lesioned and control mice. There was no increase in time spent in the dark compartment in the light-dark preference test between the groups. Similarly, there was no difference in the number of nose pokes in the hole board test between lesioned and control mice. Overall, these data suggest that the MPTP-lesioned mouse has potential to be used as an animal model of memory impairments associated with PD. On the other hand, acute treatment with MPTP does not induce significant changes in affective behavior in C57BL/6 mice.

**Publications:**

(1) Jakowec M.W., K. Nixon, E. Hogg, T. McNeill, and G. M. Petzinger (2004) Tyrosine hydroxylase and dopamine transporter expression following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurodegeneration of the mouse nigrostriatal pathway. J. Neurosci. Res. 76 (4) 539-550.

(2) Fisher, B.E., G. M. Petzinger, K. Nixon, E. Hogg, S. Bremmer, C. K. Meshul, and M. W. Jakowec (2004) Exercise-Induced behavioral recovery and neuroplasticity in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned mouse basal ganglia. Journal Neuroscience Research 77: 378-390.

(3) Jakowec, M.W., and G.M. Petzinger (2004) The MPTP-Lesioned Model of Parkinson's Disease with Emphasis on Mice and Nonhuman Primates. Comparative Medicine 54 (5) 497-513.

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(5) Petzinger, G.M., K. Nixon, B. E. Fisher, A. Abernathy, and M. W. Jakowec. (2006) Behavioral Recovery in the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-lesioned Squirrel Monkey (*Saimiri sciureus*): Analysis of Striatal Dopamine and the Expression of Tyrosine Hydroxylase and Dopamine Transporter Proteins. J. Neurosci. Res. 83: 332--347.

(6) Petzinger, G. M., and M. W. Jakowec (2007) Animal Model of Parkinson's Disease. In: Handbook of Parkinson's Disease, eds. R. Pawha and K. Lyons, Marcel Dekker, Inc., NY, NY.

(7) Petzinger, G. M., D. M. Togasaki, G. Akopian, J. P. Walsh, and M. W. Jakowec (2007) Nonhuman primate models of Parkinson's disease and experimental therapeutics. In: Parkinson's Disease: Pathogenic an Therapeutic Insights from Toxin and Genetic Models. Ed. By R. Nass and S. Przedborski, Elsevier Co., San Diego, CA.

(8) Petzinger, G. M., J. Walsh, G. Akopian, E. Hogg, A. Abernathy, P. Arevalo, P. Turnquist, B. E. Fisher, D. Togasaki, and M. W. Jakowec (2007) Effects of treadmill exercise on dopaminergic transmission in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-(MPTP)-lesioned mouse model of basal ganglia injury. Journal of Neuroscience 27 (20) 5291-5300.

(9) Manuscript in Preparation: Jon VanLeeuwen, Giselle M. Petzinger, Marta Vuckovic, Maria Ramirez, Pablo Arevalo, and Michael W. Jakowec. Altered AMPA-Receptor Expression with Treadmill Exercise in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-Lesioned Mouse Model of Basal Ganglia Injury.

**Selected Presentations:**

(1) Petzinger, Giselle "Neuroplasticity in the MPTP-lesioned Nonhuman Primate", Workshop: Plasticity and Repair in Neurodegenerative Disorders, Lake Arrowhead, California, Workshop, Feb19-22, 2004.

(2) Jakowec, Michael " The Role of Exercise in Enhancing Neuroplasticity in the MPTP-lesioned mouse", Workshop: Plasticity and Repair in Neurodegenerative Disorders, Lake Arrowhead, California, Workshop, Feb 19-22, 2004.

(3) Petzinger, Giselle "Enhancing Neuroplasticity in models of Basal Ganglia Injury", Van Der Muelen Symposium, University of Southern California, Keck School of Medicine, April 1, 2005.

(4) Petzinger, Giselle "Neuroplasticity and behavioral recovery in the MPTP-lesioned nonhuman primate" Workshop: Plasticity and Repair in Neurodegenerative Disorders, Lake Arrowhead, California, Workshop, May 11-14, 2006.

(5) Petzinger, Giselle "Exercise-enhanced motor recovery in the MPTP-lesioned mouse model of Parkinson's disease". Parkinson Study Group Annual Meeting, Chicago, IL. October 2006.

(6) Petzinger, Giselle "Exercise, neuroplasticity, and animal models of Parkinson's disease". Parkinson Disease Foundation 50$^{th}$ Anniversary Research Symposium, New York City, October 2007.

(7) Jakowec, Michael "Exercise and Parkinson's Disease", National Parkinson's Disease Research Symposium, San Diego, CA, November 2007.

**Conclusions:**

The MPTP-lesioned mouse and squirrel monkey are valuable models for investigating neuroplasticity of the injured basal ganglia. These models can serve as valuable tool to investigate the molecular mechanisms by which extrinsic factors can be applied to enhance recovery. In mice, studies in this proposal are designed to determine the role of intensive treadmill exercise in enhancing motor recovery. Meanwhile the nonhuman primate, with its exquisite parkinsonian features and similarity of anatomical features to the human condition, serves as an excellent means to examine the role of pharmacological replacement therapy targeting the dopaminergic system and the potential role in influencing recovery.

Studies in the MPTP-lesioned mouse model and exercise from the first component of this proposal indicate that intensive treadmill exercise can enhance motor behavioral recovery employing mechanisms that are different from those seen with intrinsic neuroplasticity. Our results indicate that intensive treadmill exercise in MPTP-lesioned mice leads to (i) increased motor recovery and enhanced motor learning (ii) suppression of striatal DAT and TH proteins, (iii) increased stimulus evoked dopamine release as seen in fast-scan cyclic voltammetry, (iv) differential expression of DAT and TH mRNA transcripts, (v) altered expression of specific subunits of the AMPA and NMDA receptor subtypes, and (vi) altered expression of the dopamine receptor D2.

Studies in the second component of this proposal utilizing the MPTP-lesioned nonhuman primate show that the pharmacological application through dopamine replacement therapy with Sinemet (levodopa plus carbidopa) or the D2/D3 agonist pramipexole leads to enhancement of the intrinsic neuroplasticity we observe. For example, treated MPTP-lesioned animals show (i) increased levels of striatal dopamine, (ii) increased amphetamine-evoked dopamine release using microdialysis, (iii) elevated levels of TH and DAT protein in the caudate and putamen, (iv) differential expression of DARPP-32 and its phosphorylated forms in the caudate-putamen. Electrophysiological studies have shown a shift in the AMPA/NMDA ratio, altered corticostriatal drive, shifts in the subunit composition of glutamate channels, and that LTD expression at lateral cortico-putamen synapses from the 9-month MPTP-lesioned squirrel monkey is D2 dependent. One unexpected finding was the development of dyskinesia during treatment with Pramipexole, a behavioral characteristic not yet reported in the scientific literature.

**Appendices: Attached.**

**(1) Manuscript:** Jakowec M.W., K. Nixon, E. Hogg, T. McNeill, and G. M. Petzinger. (2004) Tyrosine hydroxylase and dopamine transporter expression following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurodegeneration of the mouse nigrostriatal pathway. J. Neurosci. Res. **76** (4) 539-550.

**(2) Manuscript:** Fisher, B.E., G. M. Petzinger, K. Nixon, E. Hogg, S. Bremmer, C. K. Meshul, and M. W. Jakowec. (2004) Exercise-Induced behavioral recovery and neuroplasticity in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned mouse basal ganglia. Journal Neuroscience Research **77**: 378-390.

**(3) Manuscript:** Shakil, S. S., H. K. Homer, C. Moore, A. T. Abernathy, M. W. Jakowec, G. M. Petzinger, and C. K. Meshul (2005) High and low responders to novelty show differential effects in striatal glutamate. Synapse **58** (3) 200-207.

**(4) Manuscript:** Hughes-Davis, E. J., J. P. Cogen, M. W. Jakowec, H. W. Cheng, G. Grenningloh, C. K. Meshul, and T. H. McNeill. (2005) Differential regulation of the growth-associated proteins GAP-43 and superior cervical ganglion-10 in response to lesions of the cortex and substantia nigra in the adult rat. Neuroscience. **135** (4) 1231-1239.

**(5) Manuscript:** Petzinger, G.M., K. Nixon, B. E. Fisher, A. Abernathy, and M. W. Jakowec. (2006) Behavioral Recovery in the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-lesioned Squirrel Monkey

(*Saimiri sciureus*): Analysis of  Striatal Dopamine and the Expression of Tyrosine Hydroxylase and Dopamine Transporter Proteins. J. Neurosci. Res. 83: 332--347.

**(6) Manuscript**: Petzinger, G. M., D. M. Togasaki, G. Akopian, J. P. Walsh, and M. W. Jakowec (2007) Nonhuman primate models of Parkinson's disease and experimental therapeutics. In: Parkinson's Disease: Pathogenic and Therapeutic Insights from Toxin and Genetic Models. Ed. By R. Nass and S. Przedborski, Elsevier Co., San Diego, CA.

**(7) Manuscript:** Petzinger, G. M., J. Walsh, G. Akopian, E. Hogg, A. Abernathy, P. Arevalo, P. Turnquist, B. E. Fisher, D. Togasaki, and M. W. Jakowec (2007) Effects of treadmill exercise on dopaminergic transmission in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-(MPTP)-lesioned mouse model of basal ganglia injury. Journal of Neuroscience 27 (20) 5291-5300.

Journal of Neuroscience Research 76:539–550 (2004)

# Tyrosine Hydroxylase and Dopamine Transporter Expression Following 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine-Induced Neurodegeneration of the Mouse Nigrostriatal Pathway

**Michael W. Jakowec,* Kerry Nixon, Elizabeth Hogg, Tom McNeill, and Giselle M. Petzinger**

Department of Neurology, Keck School of Medicine, University of Southern California, Los Angeles, California

Administration of the neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to C57BL/6 mice targets nigrostriatal dopaminergic neurons, leading to cell death and the depletion of striatal dopamine. After MPTP lesioning in young adult mice, surviving nigrostriatal dopaminergic neurons display robust and reproducible return of striatal dopamine weeks to months after injury. Thus, the mouse provides an excellent model with which to investigate the mechanisms underlying neuroplasticity of the nigrostriatal system following neurotoxic injury. The purpose of this study was to analyze proteins and mRNA transcripts of genes involved in dopamine biosynthesis (tyrosine hydroxylase; TH) and uptake (dopamine transporter; DAT) with regard to time course (7–90 days) after MPTP lesioning. Molecular analysis using immunohistochemistry and Western immunoblotting techniques demonstrated an increase in striatal TH by 30–60 days postlesioning that returned to near-control (prelesioned) levels by 60–90 days. In situ hybridization histochemistry indicated that this increase in TH protein might be due in part to increased TH mRNA expression in surviving nigrostriatal dopaminergic neurons. Analysis of TH protein at 7, 30, 60, and 90 days postlesioning with two-dimensional polyacrylamide gel electrophoresis in conjunction with Western immunoblotting revealed altered TH protein isoforms migrating at isoelectric points different from those of the native isoform. In contrast to TH protein, which returned to prelesioned levels by 60 days, DAT protein analysis showed that increased expression of striatal DAT protein did not return to near-prelesion levels until 90 days postlesioning. These results suggest that TH and DAT may differ in their time course of expression in surviving dopaminergic neurons and may play a role in mediating the return of striatal dopamine.



**Key words:** MPTP; DAT; substantia nigra; striatum; Parkinson's disease; neurotoxic injury

Recent advances in our understanding of the mammalian brain have revealed that the adult central nervous system (CNS) possesses a tremendous capacity for repair following injury. Repair in the form of neuroplasticity can occur through alterations at a number of different levels within the CNS, including 1) synapse function, 2) synapse number, 3) neuronal phenotype, 4) neuronal and glial specific protein and gene patterns of expression, and 5) neuronal sprouting and branching. In our laboratory, we are interested in understanding the molecular mechanisms involved in neuroplasticity following injury with the neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Administration of MPTP to C57BL/6J mice leads to the destruction of substantia nigra dopaminergic neurons and the depletion of striatal dopamine (Jackson-Lewis et al., 1995). The degree of lesioning is such that approximately 30-35% of the nigrostriatal dopaminergic neurons survive as determined by both section sampling and unbiased stereological counting techniques. These surviving neurons may act as a template mediating recovery either with the nigrostriatal dopaminergic system or other neurotransmitter system affecting basal ganglia function. Immediately after MPTP lesioning, there is a nearly 90% reduction in the level of striatal dopamine. This reduced level of dopamine remains low for the next 30 days but begins to increase and achieves near-prelesion levels 3–4 months after injury (Ricaurte et al., 1986; Bezard et al., 2000). This phenomenon is more pronounced in young adult mice compared with aged mice, in which recovery of striatal dopamine is severely attenuated (Ricaurte et al., 1987b). The reason for this age-

*Correspondence to: Michael W. Jakowec, PhD, Assistant Professor, Department of Neurology, MCH-148, Keck School of Medicine, University of Southern California, 1333 San Pablo Street, Los Angeles, CA 90033. E-mail: mjakowec@surgery.usc.edu

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dependent effect is unclear, but it may share some features similar to the recovery seen in other injury models, such as hypoxia-ischemia and trauma.

The purpose of this study was to investigate the pattern of expression of proteins and mRNA transcripts involved in basal ganglia function, including 1) tyrosine hydroxylase (TH), which is the rate-limiting enzyme in dopamine biosynthesis, and 2) the dopamine transporter (DAT) responsible for dopamine uptake at the synapse. Young adult male C57 BL/6J mice were administered a moderately severe MPTP lesion by using a series of four injections at a concentration of 20 mg/kg, free base, over an 8-hr period. Brain tissue was harvested from the young mice in a time course fashion from 7 through 90 days after MPTP lesioning, and the levels of striatal dopamine, striatal TH and DAT, and TH mRNA transcript were determined. The extent of lesioning was determined by counting the number of substantia nigra pars compacta (SNpc) dopaminergic neurons using unbiased stereological techniques. In addition, because alteration in TH activity through phosphorylation has been proposed as a component of early biochemical compensation, we examined the posttranslational modification of TH protein by using two-dimensional (2-D) polyacrylamide gel electrophoresis in conjunction with Western immunoblotting. An understanding of the molecular mechanisms of intrinsic neuroplasticity within the injured nigrostriatal system may identify new therapeutic targets for the development of means to enhance neuroplasticity or to restore function to surviving dopaminergic neurons.

## MATERIALS AND METHODS

### Animals and MPTP Lesioning

Young adult (8 weeks old) C57BL/6 mice supplied by Jackson Laboratory (Bar Harbor, ME) were used for all experiments. MPTP (Sigma, St. Louis, MO) was administered in a series of four intraperitoneal injections of 20 mg/kg (free base) at 2-hr intervals for a total of 80 mg/kg. In the 8-week-old mouse, this regimen leads to an approximately 60–70% loss of nigrostriatal neurons (verified by using unbiased stereological methods in our laboratory) and an 80–90% depletion of striatal dopamine levels measured 7 days after lesioning (Jackson-Lewis et al., 1995). Brain tissue was collected for HPLC analysis at postlesioning days 3, 7, 21, 60, 90, and 120. Brain tissue was collected for immunohistochemical, Western immunoblot, and in situ hybridization analyses at postlesion days 7, 14, 30, 60, and 90. In each treatment group and at each time point, there were at least six mice, for a minimum total of 42 mice per time course study. The time course study was carried out in three independent runs. Saline-injected mice served as controls. Preliminary studies have shown that saline control levels did not change through the time course; therefore, collection of control tissues was carried out at injection days 0 and 30, reducing the number of animals needed in these studies.

### Determination of Striatal Dopamine Levels and Metabolites by HPLC

Neurotransmitter concentrations were determined according to an adaptation by Irwin et al. (1992) of the method of Kilpatrick and colleagues (1986). The striatum (n = 6 animals at each time point and age) was dissected, and tissues were immediately frozen in liquid nitrogen and stored at −80°C until analysis. Tissues were homogenized in 0.4 N perchloric acid, proteins were separated by centrifugation, and the supernatant was used for HPLC analysis. The protein pellet was resuspended in 0.5 N NaOH and total protein concentration determined using the BCA detection method (Pierce, Rockford, IL). Striatal dopamine was expressed as nanograms dopamine per milligram protein. Statistical analysis was carried out by using a one-way ANOVA with Dunnett's posttest comparing saline (control) treatment with MPTP-lesioned groups.

### Immunohistochemical Staining for TH and DAT Proteins

Probes used included antibodies against TH (polyclonal antibody made in rabbit or a monoclonal made in mouse; Chemicon, Temecula, CA), and DAT (monoclonal made in rat; Chemicon). Mice (6 for each treatment group and time point) designated for immunohistochemistry were administered pentobarbital (100 μl of 40 mg/ml, i.p.), then perfused transcardially with 50 ml of ice-cold 0.9% saline, followed by 50 ml of 4% paraformaldehyde/phosphate-buffered saline, pH 7.2 (called *PFA/PBS*). After perfusion, brains were quickly removed, immersion fixed in 4% PFA/PBS at 4°C for 24–48 hr, then cryoprotected in 20% sucrose for 24–48 hr. Brains were then quickly frozen in isopentane on dry ice; cut into 30-μm-thick sections, placed in phosphate-buffered saline, pH 7.2; and used immediately for immunohistochemical staining. Sections were rinsed in TBS (50 mM Tris, pH 7.2, with 0.9% NaCl); quenched in 10% methanol/3% $H_2O_2$/50 mM Tris, pH 7.2; blocked in 4% normal serum; exposed to antibody (concentration 1:1,000) for 48 hr at 4°C; rinsed in TBS; and then exposed to secondary antibody made against the species of the primary antibody (using the ABC Elite Kit; Vector, Burlingame, CA). Antibody staining was visualized by development in 0.1% diaminobenzoic acid/3%$H_2O_2$. To ensure that differences in staining intensity were in fact due to differences in antigen expression, multiple sections from each treatment group and time point were concurrently used under identical staining conditions. Specificity of antibody probes was verified by methods that eliminated staining, including 1) omitting primary antibody, 2) omitting secondary antibody, and 3) omitting both primary and secondary antibodies.

### Western Immunoblotting

Western immunoblotting was used to determine the relative striatal expression of TH and DAT protein. The immunoblotting technique was previously described (Jakowec et al., 1995a, 1998, 2001). In total, six mice were used at each time point. After decapitation, tissue was dissected from the striatum or ventral mesencephalon and homogenized in buffer [25 mM Tris, pH 7.4, 1 mM EDTA, 100 μM phenylmethylsulfonyl fluoride (PMSF)]. Protein concentration was determined by the BCA method (Pierce). Equal amounts of protein (25 μg) were separated by the method of Laemmli (1970). Proteins were transferred to nitrocellulose filters by electroblotting in Towbin buffer (Towbin et al., 1979). Filters were blocked in TS-Blotto (50 mM Tris, pH 7.4, 0.9% NaCl, 5% nonfat milk), then

primary antibody (1:2,000), and exposed to secondary antibody and visualized by chemiluminescence (Pierce). Filters were apposed to film (Hyperfilm ECL; Amersham, Arlington Heights, IL) and processed in X-Omat developer. Images were scanned into a computer using a Personal Laser Densitometer (Molecular Dynamics, Sunnyvale, CA), and the intensity of bands was determined by using computer-assisted image analysis (NIH Image). The intensities of bands from Western blot autoradiographs were expressed as relative optical density. The relative optical density of each treatment group was expressed as a percentage of the day-7 saline-injected group. Group comparisons were made by using one-way ANOVA with Bonferoni correction to determine statistical significance or effect size (meaningful difference; Thomas et al., 1991).

### Unbiased Stereological Counting of SNpc Dopaminergic Neurons

The number of nigrostriatal dopaminergic neurons in the SNpc was determined by using unbiased stereology with the computer-imaging program BioQuant Nova Prime (Bioquant Imaging, Nashville, TN) and an Olympus BX-50 microscope equipped with a motorized stage and CCD camera. Brain tissue was prepared from three mice in each group as described for immunohistochemical staining. Tissue was sliced at 30 μm thickness and every third section collected and stained for TH immunoreactivity and counterstained for Nissl substance. The SNpc was delineated from the rest of the brain based on TH immunoreactivity. Each ventral mesencephalon section was viewed at low magnification (×10 objective) and the SNpc outlined and delineated from the ventral tegmental-immunoreactive neurons by using the third nerve and cerebral peduncle as landmarks. Neurons were viewed at high magnification (×80 objective) and counted if they displayed TH immunoreactivity and had a clearly defined nucleus, cytoplasm, and nucleolus. The total number of SNpc dopaminergic neurons was determined based on the method of Gunderson (1987).

### Two-Dimensional Polyacrylamide Gel Electrophoresis/ Western Immunoblotting

Striatal tissue was homogenized in buffer (25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 μM PMSF, and 0.5% Triton X-100), and the total protein concentration was determined by the BCA method (Pierce). A total of 50 μg of protein was resuspended in isoelectric focusing buffer (8 M urea, 2% NP-40, 0.8% ampholytes, pH 3–10, 2% β-mercaptoethanol, and 100 μM PMSF) and placed over a pH 3–10 NL IPG strip (Pharmacia, Piscataway, NJ). Proteins were separated based on their isoelectric point by using the IPGphor (Pharmacia) for 60,000 volt-hours. After isoelectric focusing, the IPG strip was placed over a 12.5% polyacrylamide gel, and proteins were separated based on their molecular weight (Laemmli, 1970). A "standards well" to one side of the 2-D strip well was spiked with 25 μg of total protein (from the original tissue homogenate) to identify the TH protein migration point. After protein separation, the gel was subjected to identical conditions as described for the 1-D polyacrylamide gel electrophoresis/ Western immunoblotting (see above). Sample gels (after the second-dimension separation) were subjected to silver staining to visualize total protein pattern, ensuring adequate separation and quality (Harrington and Merril, 1984).

### In Situ Hybridization Histochemistry

In situ hybridization histochemistry (ISHH) was used to determine the relative expression of TH mRNA in the midbrain. Methods for this technique have previously been described (Jakowec et al., 1995b, 1998). At least six mice in each treatment group and time point were used. After decapitation, brain tissue was quickly removed and frozen in isopentane on dry ice. Sections were cut at 14 μm thickness on a Leica Jung 1850 cryostat, thaw mounted onto poly-L-lysine-coated microscope slides, dried on a 55°C slide warmer, and fixed in 4% paraformaldehyde/phosphate-buffered saline. Sections were dehydrated in successive ethanol washes (30%, 60%, 80%, 95%, and 100%), deprotonated in triethanolamine/acetic anhydride, delipidated in chloroform, and dehydrated in ethanol. A $^{35}$S-ribonucleotide probe was generated from a cDNA clone of the TH gene (Grima et al., 1985; Chesselet et al., 1987) by using an in vitro transcription kit from Promega (Milwaukee, WI). Tissue sections were exposed to hybridization buffer containing 4× standard sodium citrate (SSC; 1× SSC is 0.15 M NaCl, 0.015M sodium citrate, pH 7.0), 50% formamide, 1× Denhardt's solution, 250 μg/ml tRNA, 500 μg/ml salmon sperm DNA, 10% dextran sulfate, with $1 \times 10^6$ cpm of radiolabeled ribonucleotide probe. After 24 hr, tissue sections were washed in 2× SSC/50% formamide/0.1% β-mercaptoethanol at 37°C for 30 min, then in 20 μg/ml RNase in 0.5 M NaCl/10 mM Tris, pH 7.4, at 37°C for 45 min, then in 2× SSC/50% formamide/0.1% β-mercaptoethanol at 60°C for 1 hr, then in 0.1× SSC/0.1% β-mercaptoethanol at 65°C and rinsed in ethanol. Slides were placed against high-resolution film (Hyperfilm β-Max; Amersham) with radioactive standards (Amersham) for 3–7 days. The relative optical density above the SNpc from 20 different sections from groups of animals injected with saline or MPTP (at postlesioned days 7, 30, 60, and 90) was determined with a Molecular Dynamics Personal Densitometer. To minimize potential sources of variation between different experimental runs, slides from the different treatment groups and time points were processed concurrently using identical hybridization cocktail, probe concentration, probe preparation, wash regimen, and length of film exposure.

## RESULTS

### Striatal Dopamine Levels

The time course of striatal dopamine return was determined by HPLC analysis (Fig. 1). The regimen of MPTP administration used in these studies resulted in a depletion of striatal dopamine level at 3 days postlesioning to 14% ± 6% (23.4 ± 7.8 ng dopamine/mg protein; mean ± SEM) of prelesioned levels (where prelesioned levels of 100% were equal to 155.9 ± 17 ng of dopamine/mg protein). A slight decline (but not statistically significant compared with the level at 3 days post-MPTP) was seen at 7 days post-MPTP (10.3 ± 5 ng dopamine/mg protein). At 21 days postlesioning, dopamine levels remained low (22% ± 9% of control equal to 34.3 ± 12.5 ng dopamine/mg protein) but showed an

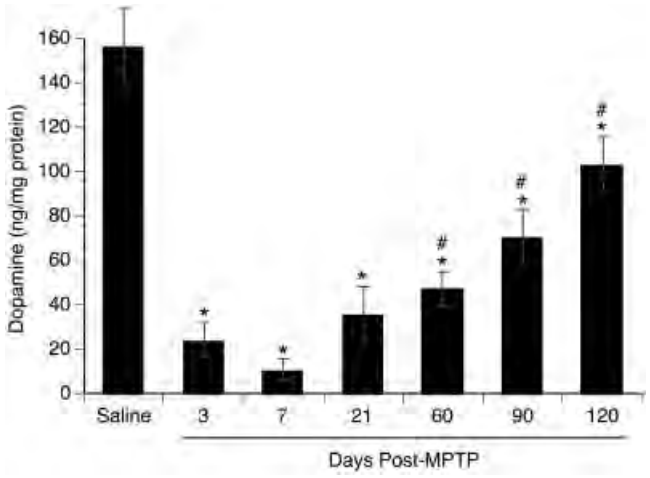


Fig. 1. Time course of striatal dopamine return after MPTP lesioning in young and old mice. The concentration of dopamine in tissue dissected from the midstriatum was determined by HPLC analysis at post-MPTP lesioning days 3, 21, 60, 90, and 120 days. Mice administered saline acted as control (day 0 time point). The concentration of dopamine is expressed as the percentage of control (saline injected) level. Asterisks represent statistical significance compared with the saline group and the number signs indicate statistical significance compared with the 7 day post-MPTP lesion groups ($P < .05$). Error bars are SEM.

increase by postlesioned day 60 (30% ± 6% of control equal to 46.8 ± 5.0 ng dopamine/mg protein). By postlesion day 90 the level of dopamine was at 45% ± 10% of control (equal to 70.2 ± 8.0 ng dopamine/mg protein) and reached 67% ± 9% of control (102.9 ± 8.0 ng dopamine/mg protein) by 120 days postlesioning. All post-MPTP lesioning dopamine levels were statistically significantly different ($P < .05$) from prelesioned (saline) control levels (at day 0). The levels of dopamine at post-MPTP lesioning days 60, 90, and 120 were statistically significantly different compared with the 7-day time point ($P < .001$). The level of dopamine in saline-treated animals did not change significantly during the time course of these studies.

## Degree of MPTP Lesioning

The degree of nigrostriatal cell loss in the SNpc following MPTP administration was determined by using an unbiased stereology counting technique. Nigrostriatal dopaminergic neurons were stained for TH immunoreactivity (TH-ir) and counterstained for Nissl substance. The saline-injected mice had a total of 23,670 (SEM = 1,250; n = 2) TH-ir neurons in the SNpc, whereas mice analyzed 7 and 30 days after MPTP lesioning had a total of 7,410 (SEM = 304; n = 2) TH-ir neurons. This represented a total decline of 69% in the number of SNpc dopaminergic neurons. Representative sections through the midbrain are shown in Figure 2, indicating that there is no significant change in the number of TH-ir neurons during the first month post-MPTP lesioning. The degree of SNpc dopaminergic neuron cell loss determined by using a lesioning regimen of four injections of 20 mg/kg MPTP (free base) is similar to that previously reported with a sampling technique to count TH-ir neurons (Jackson-Lewis et al., 1995). These results indicate that there is no substantial increase in TH-ir neurons during recovery from MPTP lesioning.

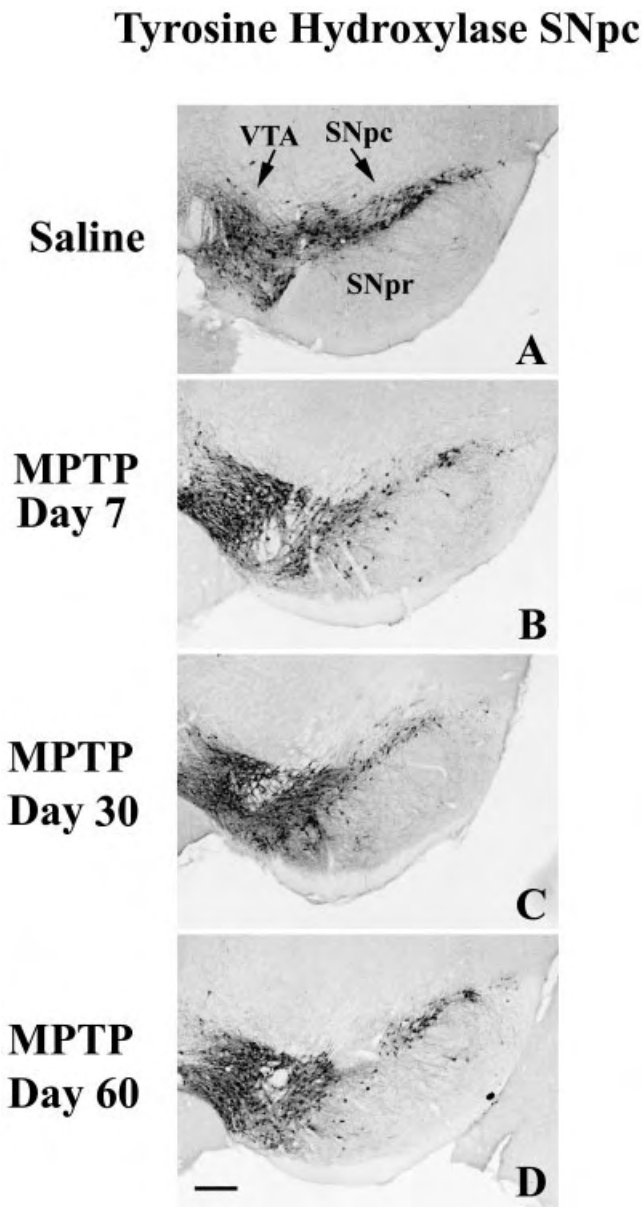


Fig. 2. Degree of MPTP lesioning in the substantia nigra pars compacta. Coronal sections stained for TH-ir at the level of Bregma –3.20 showing representative images from the midbrain of mice saline injected (**A**), 7 days post-MPTP lesioning (**B**), 30 days post-MPTP lesioning (**C**), and 60 days post-MPTP lesioning (**D**). Unbiased stereological methods were used to show that the MPTP lesioning regimen used in these studies led to an approximately 70% loss of nigrostriatal dopaminergic neurons. Scale bar = 100 μm.

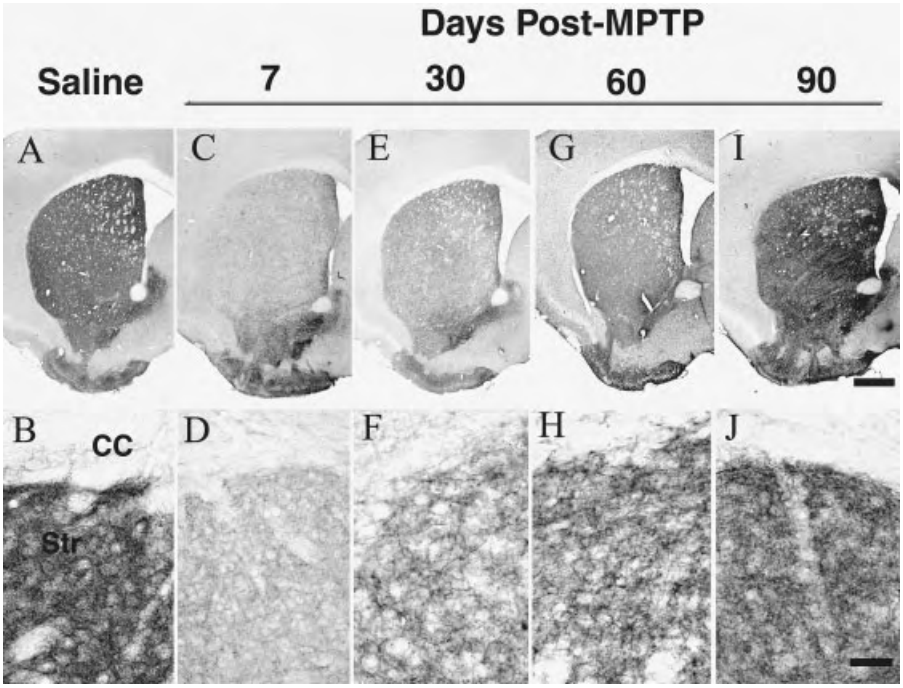


Fig. 3. TH protein expression in the striatum after MPTP lesioning. Immunohistochemistry analysis using an antibody against TH protein demonstrated increased expression that correlated with the return of striatal dopamine. Coronal sections at the level of the midstriatum (Bregma 1.00) were stained for TH protein and images made at low magnification (20×; upper panels) or high magnification (400×; lower panels). In animals administered saline, intense TH-ir was seen throughout the striatum (dark staining; **A**), and a high-magnification image (**B**) taken from the most dorsal aspect of the striatum adjacent to the corpus callosum (cc) showed a thick network of TH-positive fibers. At 7 days post-MPTP lesioning (**C**), there was a severe depletion of TH-ir, and at high magnification (**D**) only small numbers of positive immunoreactive fibers were evident. At 30 days post-MPTP lesioning (**E**), there was an increase in TH-ir in the striatum, with large fibers evident (**F**). At 60 days post-MPTP lesioning, there was a further increase in TH-ir (**G**), with increased fiber density (**H**). At 90 days, TH-ir increased (**I**) relative to 60 days, and the TH-ir fiber density appears to consist of both thick and thin fibers (**J**) forming a network similar to that seen in saline-injected animals but with an overall lower level of immunoreactivity. Scale bar in I = 100 μm for A,C,E,G,I; bar in J = 10 μm for B,D,F,H,J.

## Analysis of TH Expression

The pattern of expression of TH protein in the striatum was determined by using both immunohistochemical staining (to examine anatomical distribution of TH-ir) and Western immunoblotting (to compare relative TH protein levels at the different time points). Immunohistochemical staining of coronal sections at the level of Bregma 1.00 showed intense fibrous TH immunoreactivity throughout the striatum and olfactory tubercle of saline-injected mice (Fig. 3A,B). At 7 days post-MPTP lesioning, the degree of TH-ir was greatly reduced, with only a small number of immunopositive fibers within the striatum (Fig. 3C,D). At 30 days post-MPTP lesioning, the TH-ir increased, with more intense thick staining fibers observed (Fig. 3E,F). The degree of TH-ir protein increased through day 60, with greater staining observed in both large- and small-diameter fibers (Fig. 3G,H). At post-MPTP lesioning day 90, TH-ir was greater than that observed at day 60 but was still slightly below prelesioning staining levels (Fig. 3I,J).

Western immunoblot staining was performed on isolated striatal tissues to compare the relative amount of TH protein at time points after MPTP lesioning (Fig. 4). The day-7 saline-injected group was designated as 100%, and for comparison all other groups were normalized against this, with values expressed as mean ± SEM. Analysis of the relative amount of TH protein in the saline-injected group showed no significant change between day 7 and day 30 (100% vs. 102% ± 10%, respectively). However, 7 days following MPTP lesioning, the relative amount of TH-immunopositive protein declined to 28% ± 5% of the level in saline-injected mice ($P < .001$). At 30 days post-MPTP lesioning, TH-ir increased to 45% ± 12% ($P < .004$) of saline control levels. For TH protein levels at postlesioning days 60 and 90, the relative amount rose to 60% ± 14% and 74% ± 12% of that of saline-injected mice, respectively, and was not significantly different from that of the saline group.

Analysis of TH mRNA in the ventral mesencephalon was carried out by using ISHH. Images made at the level of the SNpc (Bregma –3.40) following ISHH with a $^{35}$S-radiolabeled probe against TH mRNA showed intense grain density above both the SNpc and VTA (Fig. 5) in saline-injected animals. For comparison, the relative

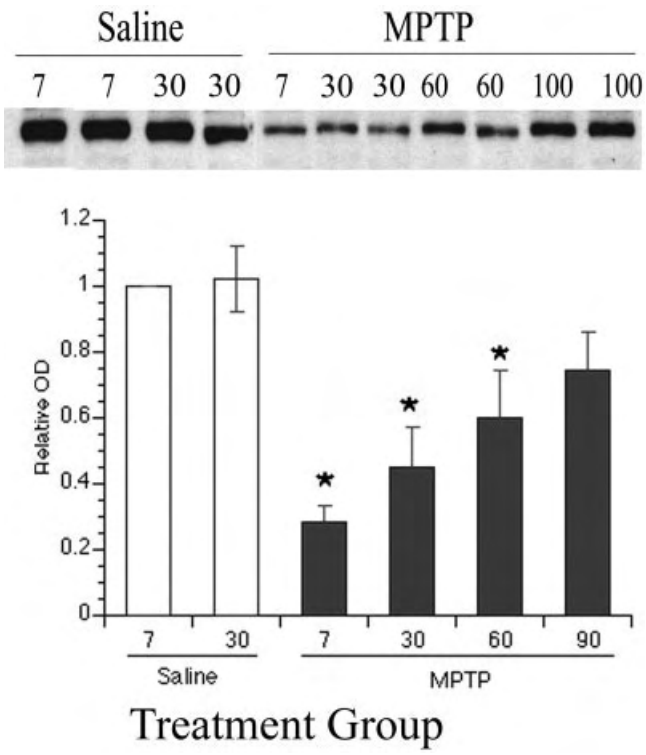


Fig. 4. Western immunoblot analysis of striatal TH protein. Analysis was carried out on tissue derived from the midstriatum of mice injected with either saline or MPTP. The upper panel shows a representative Western blot from a single experiment with equal loading of total protein. Data are expressed relative to saline-treated animals as optical density of autoradiographic bands. The relative amount of TH protein in saline-injected animals was arbitrarily set as 100%, and results from other groups were normalized against it. No difference in the relative amount of TH protein was seen after saline injection at either 7 or 30 days. MPTP lesioning resulted in a depletion of striatal TH protein at 7 days to 28% ± 5% of saline control level, increasing slightly at day 30 to 45% ± 12% of control level. A significant increase in TH protein was seen between day 30 and day 90 to 60% ± 14% and 74% ± 12% of prelesion control, respectively. Asterisks represent statistical significance compared with the saline group ($P < .05$). Error bars are SEM.

optical density for the saline group was set to 100% and the relative optical density for all other groups normalized against it. At 7 days postlesioning, the intensity of grains above the SNpc was reduced by 76% compared with saline control (from 100% ± 12.4% in the saline group to 24.0% ± 4.0% at post-MPTP lesioning day 7; Fig. 5B,C). At 30 days postlesioning, there was a slight increase in TH mRNA labeling to 31.6% ± 3.9% of control that further increased at 60 days postlesioning to 40.4% ± 5.0% of saline control (Fig. 5D,E) and by 90 days postlesioning (Fig. 5F) labeling returned to a level not significantly different from prelesioning levels (100.4% ± 8.4%). The level of mRNA increased to near-prelesion levels by day 90 post-MPTP lesioning, even though the total number of SNpc dopaminergic neurons remained near 30% of prelesion levels.

Two-dimensional PAGE separates proteins in the first dimension based on their isoelectric point and in the second dimension based on their molecular weight. Figure 6 shows the results of analysis of striatal TH protein from saline and post-MPTP lesioning days 7, 30, 60, and 90. In saline-treated mice, TH-ir is localized to the native isoelectric point (pI = 5.8). At 7 days postlesioning, the native isoform of TH-ir is nearly absent, with a small amount of TH-ir protein migrating to a new isoelectric point (Fig. 6B). At 30 and 60 days postlesioning, the amount of TH-ir protein at the new isoelectric point continues to increase as well as the TH-ir protein at the native isoelectric point (Fig. 6C,D). By 90 days postlesioning, the remaining TH-ir protein shifted toward the native isoelectric point (Fig. 6E). The apparent molecular weight (56 kDa) of the TH-ir band does not appear to change significantly during the time course of recovery.

### Analysis of DAT

The pattern of expression of the DAT protein was determined by using both immunohistochemistry (to examine anatomical distribution) and Western immunoblotting (to compare relative TH protein levels at the different time points) at the level of the midstriatum (Bregma 1.00). Immunohistochemical staining showed intense DAT-ir throughout the midstriatum (Fig. 7A,B). At 7 days postlesioning, the level of DAT-ir was greatly reduced compared with that of saline-injected mice (Fig. 7C,D). At postlesion days 14 (Fig. 7E,F) and 30 (Fig. 7G,H), there was an increase in DAT-ir compared with postlesion day 7 because of an increase in the DAT-ir fiber density. At postlesion days 60 (Fig. 7 I,J) and 90 (Fig. 7K,L), the level of DAT-ir continued to increase relative to the 30-day MPTP lesion time point and resembled the level of DAT-ir seen in saline-injected mice.

Western immunoblotting was used to determine the relative expression of DAT protein in striatal tissue during the time course of recovery. For a relative comparison, the level of DAT protein in striatal tissue homogenates from saline-injected mice was set as 100%, and the value for all other groups was normalized to it (see Fig. 8). There was no change in the relative amount of DAT protein in saline-injected mice at days 7 and 30 (100% vs. 90% ± 5%, respectively). A significant ($P < .0001$) depletion of DAT protein to 34% ± 5% of that of saline-injected mice was seen at 7 days postlesioning. At 30 days postlesioning, DAT protein increased to 44% ± 6% of that of the saline-injected group ($P < .0001$). DAT-ir continued to increase through postlesion day 60 (to 62% ± 7% of that in the saline group; $P < .001$) and day 90 (78% ± 13% vs. saline; not significantly different). Western immunoblotting with tissues from the ventral mesencephalon also showed a reduction in DAT-ir at days 7 and 30 postlesioning that returned to nearly the saline-injected levels from 60 days onward (data not shown).

## DISCUSSION

After neurotoxic injury with MPTP in the C57 BL/6 mouse, surviving nigrostriatal dopaminergic neurons

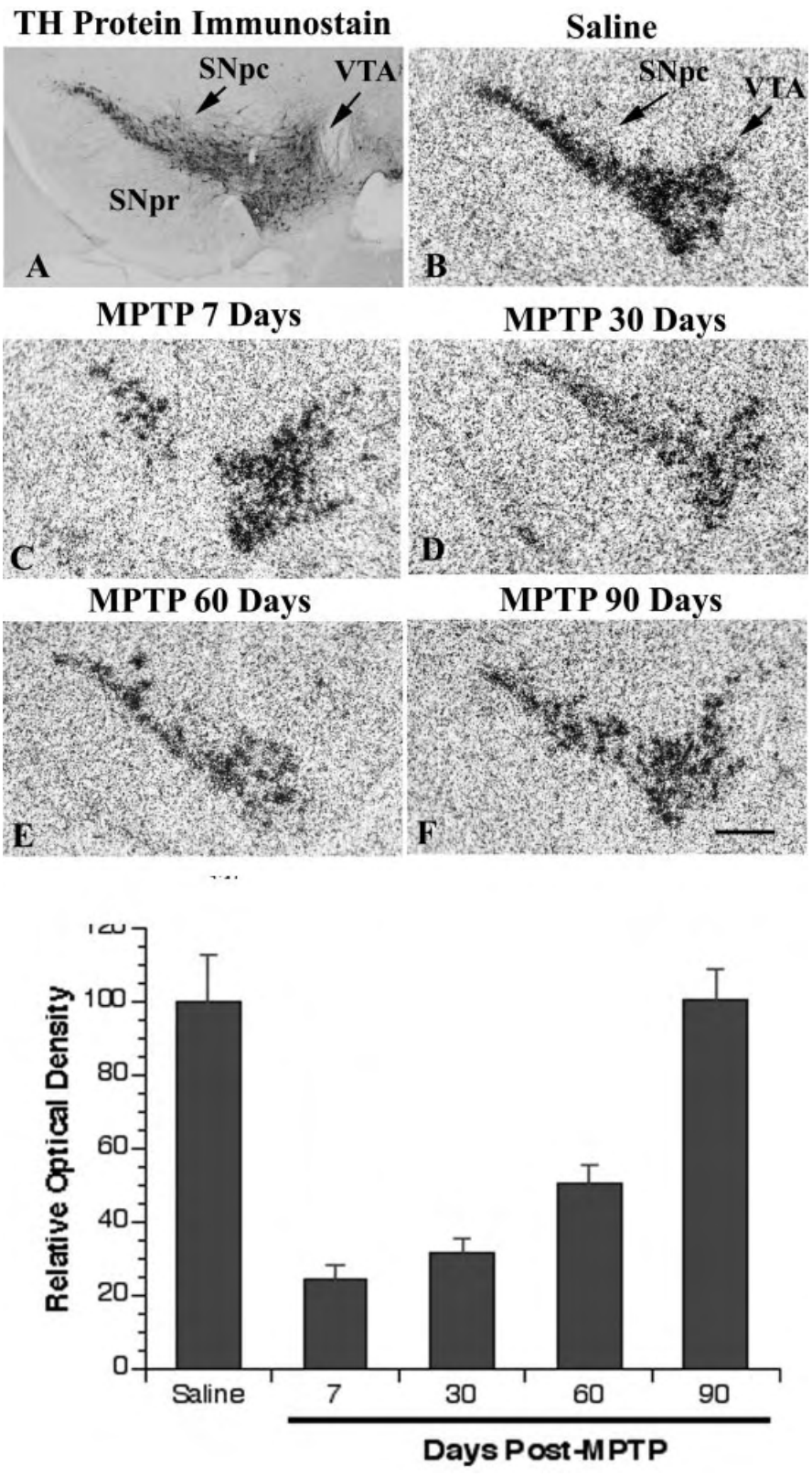


Fig. 5. TH mRNA expression after MPTP lesioning. In situ hybridization histochemistry was carried out on coronal tissue sections at the level of the midsubstantia nigra (Bregma –3.20) from saline-treated and post-MPTP lesioning days 7, 30, 60, and 90. **A:** Immunohistochemical staining against TH protein identified the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA), where TH-ir cell bodies and their terminals stain dark. **B:** In saline-injected animals, the expression of TH mRNA was robust, with a high density of autoradiographic grains seen above the SNpc and VTA. **C:** At 7 days after MPTP lesioning, there was a loss of grain intensity above the SNpc and only a slight reduction in intensity above the VTA. **D:** The SNpc grain intensity remained low through day 30. **E:** At 60 days after MPTP lesioning, there was increased grain density in the SNpc. **F:** At 90 days after MPTP lesioning, there was an increase in the autoradiographic grain intensity in the SNpc and VTA. MPTP lesion days 7, 30, and 60 are statistically significant (at $P < .001$) compared with saline controls. The optical density was not significantly different between saline and post-MPTP lesion day 90. Scale bar = 100 μm.

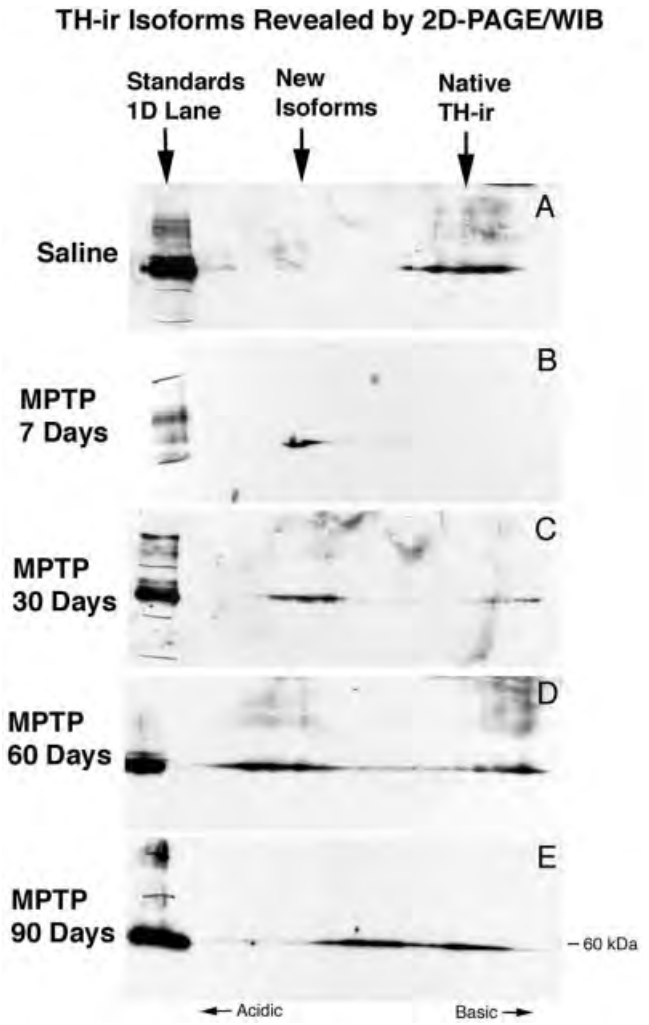


Fig. 6. TH protein expression analyzed by 2-D PAGE. Protein extracts from the striatum of both saline and MPTP-lesioned animals were examined using 2-D PAGE in conjunction with Western immunoblotting. The protein standards lane was "spiked" with striatal protein extract to locate TH-ir. **A:** Saline-injected animals showed a high degree of TH-ir as a single major band in the 1-D lane that was present in a localized cluster of several equivalent-molecular-weight spots according to 2-D separation. **B:** Seven days after MPTP lesioning, the level of TH-ir was significantly reduced as detected by both 1-D and 2-D separation. Small spots of reactivity were evident toward the acid end of the 2-D PAGE. **C:** At 30 days after MPTP lesioning, there was a return of TH-ir as seen on 1-D PAGE. By 2-D PAGE, the increase in intensity was greater at the new isoform position, with only a slight increase in intensity at the native 2-D PAGE position. **D:** At 60 days postlesioning, TH-ir continued to increase at the position of both the new isoform and the native TH position. **E:** At 90 days postlesioning, immunolabeling corresponded to both the native and the new isoform positions.

display robust and reproducible neuroplasticity. Young mice (8 weeks of age) showed return of striatal dopamine as determined by HPLC to near-prelesion levels 3–4 months after injury. Our results confirm the return of striatal dopamine described in earlier studies (Ricaurte et al., 1987b). The degree of nigrostriatal injury may only delay intrinsic neuroplasticity, in that the return of striatal TH protein or TH fiber density has been demonstrated in both mild and severe MPTP lesioning regimens (Bezard et al., 2000). The age-dependent effects of MPTP have also been demonstrated in mice and in nonhuman primates (Ricaurte et al., 1987a; Song and Haber, 2000) as well as with 6-hydroxydopamine in rodents (Onn et al., 1986; Blanchard et al., 1995, 1996).

One mechanism responsible for mediating the return of striatal dopamine in surviving nigrostriatal dopaminergic neurons is through increased expression of TH protein, the rate-limiting enzyme in dopamine biosynthesis (Hefti et al., 1980; Onn et al., 1986; Blanchard et al., 1995, 1996). Based on both immunohistochemical staining for TH protein in the striatum and Western immunoblot analysis of TH protein after MPTP lesioning, the increase in striatal dopamine correlates with the increase in striatal TH protein. Other reports have also examined the return of striatal TH protein following neurotoxic injury of the nigrostriatal pathway. In general, TH protein return occurs late (months) after 6-OHDA lesioning (Onn et al., 1986; Blanchard et al., 1995, 1996) or after MPTP lesioning in both the mouse (Bezard et al., 2000) and the nonhuman primate (Song and Haber, 2000). The time course for the return of striatal TH protein is dependent on the degree of lesioning (a threshold effect); severe lesioning fails to demonstrate return until several months postlesioning, whereas mild lesioning shows recovery within weeks (Bezard et al., 2000; Finkelstein et al., 2000; Song and Haber, 2000). Changes in TH mRNA expression after nigrostriatal injury have been less clear. For example, there are reports of mRNA increasing (Blanchard et al., 1995, 1996) and reports of it decreasing or remaining unchanged (Pasinetti et al., 1989, 1992; Sirinathsinghji et al., 1992). These discrepancies may be due to differences in degree of lesioning, time course, mode of analysis, and rodent strain used. In human parkinsonism, there is a decrease in TH protein and mRNA relative to control brains, which may reflect the severity of disease progression (Javoy-Agid et al., 1990; Kastner et al., 1993). Results from our studies indicate that the remaining nigrostriatal neurons in the midbrain increase mRNA levels such that the absolute level of mRNA labeling as determined by in situ hybridization histochemistry returns to near-prelesioned levels by 90 days. It appears that the remaining cells (30% of saline control) significantly increase the per cell level (possibly by a threefold increase) of the TH mRNA transcript. Studies are underway to quantify this observation more accurately.

In this report, we describe an early and persistent posttranslational modification of TH protein correlating with the return of striatal dopamine after MPTP lesioning in the C57 BL/6 mouse. Our results using 2-D PAGE indicated expression of an altered isoform of TH protein, having a shift in its isoelectric point with little detectable change in the apparent gross molecular weight (Gutierrez

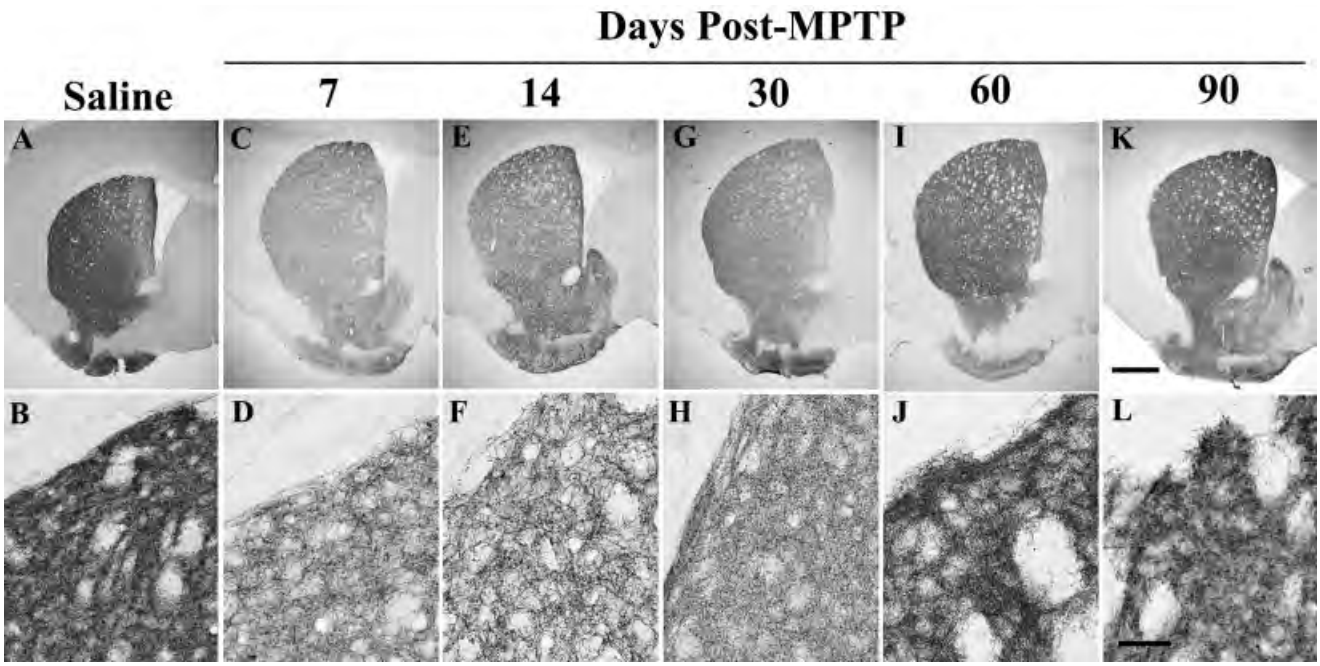


Fig. 7. Immunocytochemical analysis of DAT protein in the striatum after MPTP lesioning. Tissue sections in the coronal plane through the midstriatum (Bregma 1.00) were stained with an antibody against the DAT protein. Images were made at low magnification (20×; upper panels) to show an entire coronal hemisphere and at high magnification (400×; upper panel) from the uppermost dorsal-lateral quadrant of the striatum apposing the corpus callosum). Saline-injected mice showed intense DAT-ir throughout the striatum (**A**) and as a thick fibrous web (**B**). MPTP administration results in the depletion of DAT-ir as seen at postlesion day 7 (**C,D**). DAT-ir remained low at day 14 (**E,F**), with thick processes stained. DAT-ir increased slightly at day 30 (**G,H**) and continued to increase at day 60 (**I,J**) and day 90 (**K,L**). Scale bar in K = 100 μm for A,C,E,G,I,K; bar in L = 10 μm for B,D,F,H,J,L.

et al., 1988). This altered isoform, which was the predominant form of TH protein, occurred early (at 7 days postlesioning) and persisted (for at least 90 days postlesion). Although the molecular basis for this altered form of TH protein has yet to be determined, reports in the literature suggest phosphorylation by protein kinases, including protein kinase A, CAM-protein kinase II, and ERK (MAP kinase) as likely candidates (Campbell et al., 1986; Le Bourdelles et al., 1991; Sutherland et al., 1993; Alterio et al., 1998). Posttranslational phosphorylation of TH protein (at serine residues 8, 19, 31, and 40) leads to an increase in TH enzymatic activity, protein stability, and translocation (Zigmond et al., 1989). This increases the affinity of TH for its cofactor (biopterin) and decreases its feedback inhibition by dopamine (Zigmond et al., 1989; Haycock and Haycock, 1991; Haycock et al., 1998; Fitzpatrick, 1999). We cannot exclude nitrosylation of tyrosine residues in TH as an early effect of MPTP lesioning (Ara et al., 1998). Future studies will examine the effect of this posttranslational modification on the activity of TH enzyme to determine whether alteration in Vmax (Zigmond et al., 1989; Fitzpatrick, 1999) or "homospecific activity" (defined as TH enzymatic Vmax relative to TH protein content; Lloyd et al., 1975; Acheson and Zigmond, 1981; Zigmond et al., 1984; Onn et al., 1986; Mogi et al., 1988) are altered and whether these alterations persist throughout the recovery process.

Another mechanism for increasing synaptic availability of dopamine is through reduced uptake (Rothblat and Schneider, 1999). The dopamine transporter (DAT) protein is responsible for the reuptake of dopamine from the extracellular space for vesicular repackaging in the presynaptic terminal (Hoffman et al., 1998). Our results indicated an early decline of DAT protein (7 days after MPTP lesioning), with a gradual return to 80% of the prelesioned level by 90 days. A consequence of the early decline of DAT protein is to increase the effective synaptic concentration of dopamine (Eberling et al., 1999; Kilbourn et al., 2000). However, it is more likely that the initial decline in DAT protein is due simply to the loss of nigrostriatal fibers as a consequence of MPTP lesioning. The later increase in DAT protein correlated with the return of striatal dopamine and striatal TH protein and may serve as a marker of terminal recovery (sprouting and branching) after 30 days postlesioning. These results are consistent with the expression of genes and proteins involved in neuronal sprouting and support a morphological component to recovery. Studies in our laboratory and the laboratories of others have shown that expression of the growth-associated protein of 43 kDa (GAP-43) involved in neuronal sprouting increases after 30 days post-MPTP lesioning (Jakowec, 1999; Song and Haber, 2000).

Other factors that may influence the return of striatal TH protein include 1) neurogenesis within the substantia

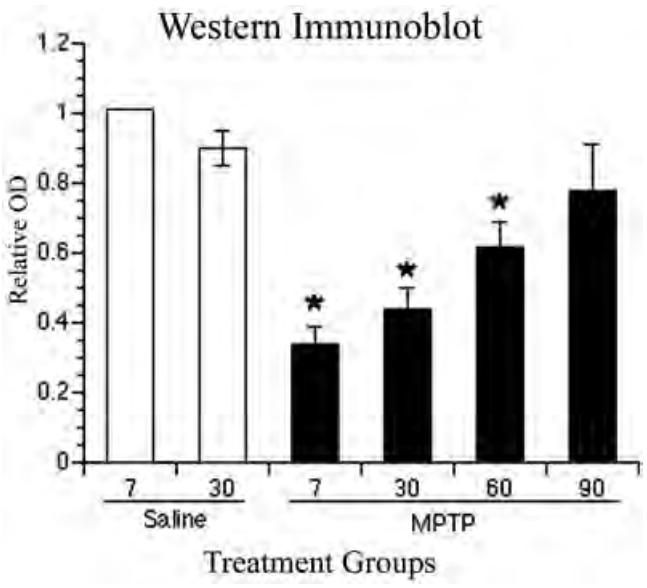


Fig. 8. Western immunoblot analysis of DAT protein in the striatum after MPTP lesioning. The expression of DAT protein was determined by using Western immunoblotting on tissue from the striatum of either saline- or MPTP-injected animals. For relative comparison, the day-7 saline-injected group was arbitrarily set at 100%, and the results from all other groups were normalized against it. There was no significant difference between saline groups at days 7 and 30. After MPTP lesioning, there was a reduction in the level of DAT protein to 34% ± 5% of control. DAT protein levels increased at 30 days postlesioning to 44% ± 6% of saline control and continued to increase at 60 and 90 days to 62% ± 7% and 78% ± 13% of control, respectively. Asterisks represent significant different from saline group ($P < .05$). Error bars show SEM.

nigra as demonstrated by bromodeoxyuridine (BrdU) labeling (Kay and Blum, 2000), 2) conversion of neurons intrinsic to the basal ganglia to a dopamine-producing phenotype (Betarbet et al., 1997), or 3) dopaminergic innervation from areas other than the substantia nigra (Ho and Blum, 1998). In this study, there was no detectable change in the number of SNpc neurons with recovery as measured with unbiased stereological techniques to determine the number of remaining dopaminergic neurons. This finding is supported by studies from other investigators (Bezard et al., 2000). Therefore, it appears that nigrostriatal dopaminergic neurons that survive the MPTP lesioning increase TH and DAT protein and may repopulate the striatum with axonal regrowth and branching.

Understanding the inherent neuroplasticity of the basal ganglia in both normal and injured states (MPTP lesion) will provide valuable information in the development of new therapeutic strategies for neurodegenerative diseases such as Parkinson's disease, traumatic brain injury, and normal aging. Because 40–50% of the nigrostriatal dopaminergic neurons still remain in autopsied specimens of individuals affected with idiopathic Parkinson's disease, therapeutic strategies may be targeted toward restoring the functional capacity of these surviving neurons (Bernheimer et al., 1973; Pakkenberg et al., 1991). Potential strategies may include 1) increasing TH gene expression through modulating transcription factors, 2) increasing TH activity through elevated kinase activity, and 3) increasing neuronal sprouting and branching through neurotrophic factors. In addition to affecting TH activity, the posttranslational modifications of TH may influence its stability, translocation rate, and terminal localization (Jarrott and Geffen, 1972). Finally, understanding the intrinsic plasticity of the basal ganglia has important implications for the role of stem cell technology in Parkinson's disease as a modulator of neuroplasticity as well as an alternative source of dopamine.

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# Exercise-Induced Behavioral Recovery and Neuroplasticity in the 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine-Lesioned Mouse Basal Ganglia

**Beth E. Fisher,[2] Giselle M. Petzinger,[1] Kerry Nixon,[1] Elizabeth Hogg,[1] Samuel Bremmer,[3] Charles K. Meshul,[3,4] and Michael W. Jakowec[1]***

[1]Department of Neurology, University of Southern California, Los Angeles, California
[2]Department of Biokinesiology and Physical Therapy, University of Southern California, Los Angeles, California
[3]Department of Behavioral Neuroscience, Oregon Health and Science University, Portland, Oregon
[4]VA Medical Center, Portland, Oregon

Physical activity has been shown to be neuroprotective in lesions affecting the basal ganglia. Using a treadmill exercise paradigm, we investigated the effect of exercise on neurorestoration. The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned mouse model provides a means to investigate the effect of exercise on neurorestoration because 30–40% of nigrostriatal dopaminergic neurons survive MPTP lesioning and may provide a template for neurorestoration to occur. MPTP-lesioned C57 BL/6J mice were administered MPTP (four injections of 20 mg/kg free-base, 2 hr apart) or saline and divided into the following groups: (1) saline; (2) saline + exercise; (3) MPTP; and (4) MPTP + exercise. Mice in exercise groups were run on a motorized treadmill for 30 days starting 4 days after MPTP lesioning (a period after which MPTP-induced cell death is complete). Initially, MPTP-lesioned + exercise mice ran at slower speeds for a shorter amount of time compared to saline + exercise mice. Both velocity and endurance improved in the MPTP + exercise group to near normal levels over the 30-day exercise period. The expression of proteins and genes involved in basal ganglia function including the dopamine transporter (DAT), tyrosine hydroxylase (TH), and the dopamine D1 and D2 receptors, as well as alterations on glutamate immunolabeling were determined. Exercise resulted in a significant downregulation of striatal DAT in the MPTP + exercise compared to MPTP nonexercised mice and to a lesser extent in the saline + exercised mice compared to their no-exercise counterparts. There was no significant difference in TH protein levels between MPTP and MPTP + exercise groups at the end of the study. The expression of striatal dopamine D1 and D2 receptor mRNA transcript was suppressed in the saline + exercise group; however, dopamine D2 transcript expression was increased in the MPTP + exercise mice. Immunoelectron microscopy indicated that treadmill exercise reversed the lesioned-induced increase in nerve terminal glutamate immunolabeling seen after MPTP administration. Our data demonstrates that exercise promotes behavioral recovery in the injured brain by modulating genes and proteins important to basal ganglia function.


**Key words:** tyrosine hydroxylase; dopamine transporter; dopamine receptors; glutamate; MPTP; Parkinson's disease

It has been established that neuroplasticity, as characterized by neurogenesis, synaptogenesis, and molecular adaptations, exists in the human nervous system. Animal models of brain injury have provided a means to both investigate and manipulate neuroplasticity. A heightened area of interest is the role that exercise plays in facilitating neuroplasticity in either the noninjured or injured brain (Fisher et al., 2001). Studies employing a variety of animal models of injury have shown that exercise can promote neuroplasticity and behavioral recovery in the hippocampus, cortex, and spinal cord (Kempermann et al., 2000). In

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*Correspondence to: Michael W. Jakowec, PhD, Department of Neurology, University of Southern California, 1333 San Pablo Street. MCH-148, Los Angeles, CA 90033. E-mail: mjakowec@surgery.usc.edu

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rodent models of basal ganglia injury, exercise has been shown to be neuroprotective (Tillerson et al., 2001, 2003; Tillerson and Miller, 2002). By restraining the unimpaired limb immediately after injury and forcing use of the impaired upper limb, behavioral and neurochemical sparing were demonstrated in the 6-hydroxydopamine (6-OHDA)-lesioned rat. This suggested that forced use of the impaired limb protected dopaminergic cells from the neurotoxic effects of 6-OHDA. In addition, Tillerson et al. (2003) reported behavioral improvement after treadmill exercise in two rodent models of basal ganglia injury (the 6-OHDA rat and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [MPTP]-lesioned mouse) (Tillerson et al., 2003). Immediate exposure to treadmill training within 12 hr of injury was associated with attenuation of dopamine loss. The investigators concluded that exercise might work largely through neuroprotective mechanisms because exercise was started within 12 hr of lesioning, and MPTP and 6-OHDA may take several days to complete cell death (Sauer and Oertel, 1994; Jackson-Lewis et al., 1995). The implication of these neuroprotective studies is that exercise may be helpful in delaying or preventing Parkinson's disease in healthy individuals (Sasco et al., 1992).

The interest in our laboratory is to investigate the role of exercise in promoting repair of the injured basal ganglia. We define this as neurorestoration, which is the capacity of surviving dopaminergic neurons to adapt after injury with potential behavioral benefits. The MPTP-lesioned mouse model of basal ganglia injury provides a means to investigate neurorestoration because 30–40% of the substantia nigra pars compacta (SNpc) dopaminergic neurons survive the lesioning regimen. Despite a 90% loss of striatal dopamine, these mice display robust and reproducible return of striatal dopamine, tyrosine hydroxylase (TH) protein, and dopamine transporter (DAT) protein 2–3 months after MPTP lesioning. This suggests that surviving nigrostriatal dopaminergic neurons provide a template for neurorestoration and would therefore provide a means to investigate the effect of exercise on facilitating neurorestoration. In addition, MPTP-lesioned mice manifest motor behavioral deficits that can be monitored throughout the recovery process (Tillerson et al., 2003). The purpose of our study was to investigate the effect of treadmill exercise on neurorestoration using the MPTP-lesioned mouse model of basal ganglia injury by introducing exercise 4 days after MPTP lesioning, a time point well after cell death is complete. Because TH, DAT, dopamine (D1 and D2) receptors and glutamate storage have been shown to be altered in the MPTP-lesioned mouse model (Jakowec et al., 2004), we chose to examine these same parameters in our MPTP exercise paradigm. Exercise was continued for 30 days to parallel the 30-day recovery period when TH is beginning to return.

## MATERIALS AND METHODS

### Housing and Acclimation of Mice

Young adult (8–10 weeks old) male C57BL/6J mice supplied from Jackson Laboratory (Bar Harbor, ME) were used for this study. There were four treatment groups: (1) saline injected; (2) saline + exercise; (3) MPTP lesioned; and (4) MPTP lesioned + exercise. Three cohorts of mice, consisting of four groups of 10 mice/group were used (total $n$ + 120 mice). Animals were housed six to a cage and acclimated to a 12-hr shift in light/dark cycle so that exercise occurred during the animals' normal wake period.

### MPTP Lesioning

MPTP (Sigma, St. Louis, MO) was administered in a series of four intraperitoneal injections of 20 mg/kg (free-base) at 2-hr intervals for a total administration of 80 mg/kg. This regimen leads to a 60–70% loss of nigrostriatal neurons (as determined by unbiased stereologic techniques for both TH staining and Nissl substance in our laboratory) and an 80–90% depletion of striatal dopamine levels (Jackson-Lewis et al., 1995). Using this regimen, nigrostriatal cell loss is complete by Day 3 after MPTP administration and persists beyond 30 days post-lesioning as determined by either unbiased stereologic counting techniques (Petzinger et al., in preparation) or section-sampling techniques (Jackson-Lewis et al., 1995).

### Selection of Mice and Exercise Protocol

Figure 1A outlines the experimental design of animal groups. Before MPTP lesioning, a baseline treadmill running assessment was conducted to insure that all animals performed similarly on the treadmill task before MPTP lesioning. Forty animals that could maintain a forward position on the 2.5-m treadmill belt for 5 min at 10 m/min were assigned randomly to the four groups. A non-noxious stimulus (metal-beaded curtain) was used as a tactile incentive to prevent animals from drifting back on the treadmill. Consequently, shock-plate incentive was not used and stress related to the activity was minimized. Exercise was initiated 4 days after saline or MPTP lesioning. For exercise training, a motorized, 10-lane rodent treadmill (Fig. 1B) was used at incremental speeds to a goal treadmill speed between 20.5–23.0 m/min (Fukai et al., 2000). All 10 mice from each of the two exercise groups (MPTP + exercise and saline + exercise) were run at the same time in the 10-lane treadmill (see Fig. 1B). Exercise duration was incrementally increased to reach the goal duration of 2 × 30 min/day (60 min), 5 days/week (with a 2-min warmup period) for a total of 30 days of exercise. Treadmill speed for each group was increased when all 10 mice within each group maintained a forward position on the 2.5-m treadmill belt for 75% of the running period. When all 10 mice within each of the two running groups maintained a forward position 100% of the time, duration for that group was increased. Mice were exercised as a group of 10 for two 30-min sessions (total 1 hr) per day with a 30-min rest period between sessions. To control for any non-exercise effects of treadmill running (handling, novel environment, noise, and vibration) nonexercised groups were placed on the top of the treadmill apparatus for a time period equivalent to

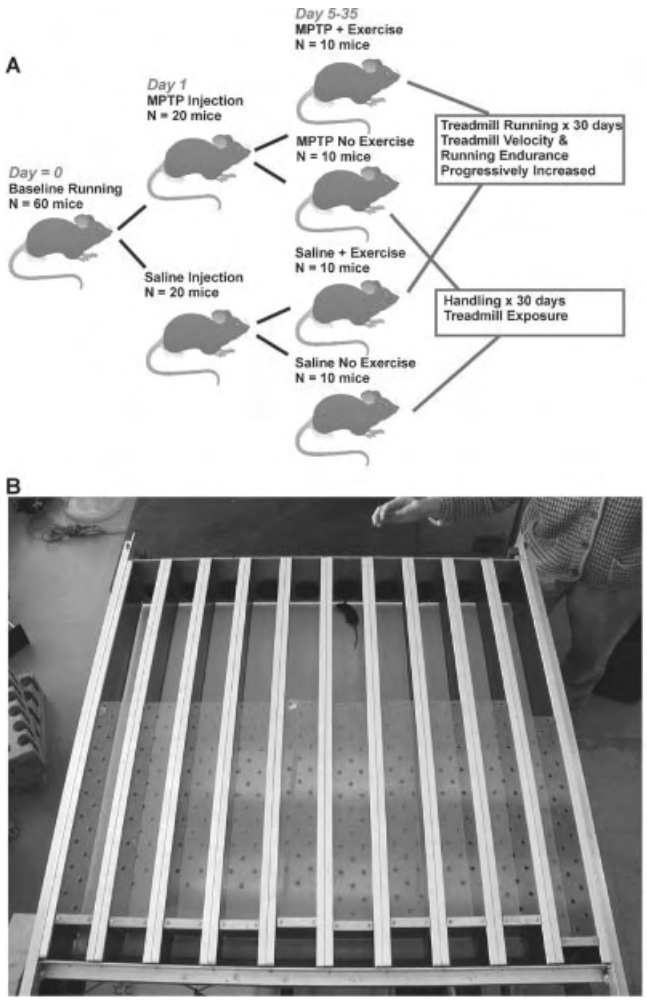


Fig. 1. **A:** Summary of experimental design with group assignment and exercise protocol. **B:** The 10-lane motorized treadmill used for exercising the mice.

exercise training (Fukai et al., 2000; Kojda et al., 2001). At the end of the 30-day running period, all animals from the four groups (exercise and non-exercise, with and without MPTP) were run to compare running speed capability. Initial treadmill velocity was set at the same speed at which initial pre-exercise baseline running capability was determined (i.e., 10 m/min). Maximum velocity for each group was defined as the velocity at which the mice, as a group of 10, could maintain a forward position on the treadmill for 75% of a 5-min running trial.

### Collection of Brain Tissue

Brain tissue was collected at 4 days post-MPTP lesioning to examine the reduction in the degree of DAT and TH immunoreactivity. Brain tissue was collected from all groups at the conclusion of the 30 days of treadmill exercise, (35 days post-MPTP lesioning). Tissue for immunohistochemical analysis was fixed by transcardial perfusion with 50 ml of ice-cold saline followed by 50 ml of 4% paraformaldehyde/phosphate-buffered saline (PFA/PBS) pH 7.2. Brains were removed, post-fixed in 4% PFA/PBS for 48 hr, cryoprotected in 20% sucrose for 24 hr, and then quickly frozen in isopentane on dry ice. Tissues for Western immunoblotting and in situ hybridization were harvested fresh after cervical dislocation. All procedures used in these studies adhered to the guidelines of the Institutional Animal Care and Use Committee (IACUC) and the National Institutes of Health. To maximize the utilization of brain tissues each technique involving either fresh tissues (for Western immunoblotting), quick frozen tissues (for in situ hybridization histochemistry), or perfusion fixed (for immunoelectron microscopy or immunohistochemistry) consisted of at least 6 to as many as 10 mice from each group in a single experimental cohort. This assured a large enough *n* to detect changes within each experimental design.

### Immunohistochemistry

Fixed tissue from at least six mice from each group was cut at 30-μm thickness, placed in phosphate buffer, and used immediately for immunohistochemistry. Commercially available antibodies included rabbit polyclonal anti-TH (Chemicon, Temecula, CA), and mouse monoclonal anti-DAT (Chemicon). Tissue sections were washed in Tris-buffered saline (TBS; 50 mM Tris pH 7.4 and 0.9% NaCl) and exposed to antibody (1:1,000) for 48 hr at 4°C. Sections were washed in TBS, and exposed to horseradish peroxidase (HRP)-conjugated secondary antibody using the ABC Elite kit (Vector Labs, Burlingame, CA). Antibody staining was visualized by development in DAB/$H_2O_2$. To ensure that differences in staining intensity were due to differences in antigen expression, multiple sections from each of the different treatment groups were handled concurrently in identical staining conditions. Control experiments excluding either primary antibody or secondary antibody were also carried out to verify staining specificity. Determination of the relative expression of TH immunoreactivity (ir) and DAT-ir in the striatum using immunohistochemistry from different mouse groups was based on published validity studies (Burke et al., 1990). For image analysis, three or four animals per treatment group and 10–12 sections per animal, spanning the midstriatum rostral to the anterior commissure (Bregma 0.25–1.25 mm) were used. Striatal images were captured at low magnification and digitized. The relative optical density (OD) (expressed as arbitrary units within the linear range of detection) of the dorsal lateral striatum was determined by subtracting the relative optical density of the corpus callosum as background. To ensure that the gray values represented an OD within the nonsaturated range of the image analysis, a Kodak Photographic step tablet (density rage to 255 OD units) captured by the CCD camera was used. Maximal tissue immunostaining relative OD units did not exceed the relative OD units of the tablet.

### Western Immunoblotting

Tissue for Western blot analysis was dissected from the mid-striatum (a 3-mm thick section between Bregma 0.00–1.50) of at least six mice from each group and homogenized in buffer (25 mM Tris pH 7.4, 1 mM EDTA, 100 μM phenylmethylsulfonylfluoride [PMSF]). Protein concentration was determined by the BCA method (Pierce, Inc.). Proteins (10 μg) were

separated by polyacrylamide gel electrophoresis (PAGE) using the method of Laemmli (1970) and transferred to nitrocellulose filters by electroblotting in Towbin buffer (Towbin et al., 1979). Filters were blocked in TS-Blotto (50 mM Tris pH 7.4, 0.9% NaCl, 5% nonfat milk) for 1 hr, then exposed to primary antibody (1:2,000) in TS for 2 hr. Filters were then washed in TS, and exposed to secondary antibody in TS-Blotto for 1 hour. After a final wash in TS, antibody binding was visualized by chemiluminescence (Pierce, Inc.) and apposing filters to film (Hyperfilm ECL; Amersham) and processed in X-OMAT developer. Images were scanned and the relative OD of bands (expressed as arbitrary units within the linear range of film) was determined using Bioquant Nova Prime, a computer-assisted image analysis program (Bioquant Imaging, Nashville, TN).

### Electron Microscopy/Immunocytochemistry

Electron microscopic immunolabeling for glutamate was carried out on mice from the saline group ($n$ + 6), saline + exercise group ($n$ + 10), MPTP group ($n$ + 7), and MPTP + exercise group ($n$ + 9). Anesthetized mice were perfused transcardially with 6 ml of heparin (1,000 U/ml) in HEPES buffer (pH 7.3) followed by 50 ml of 2.5% glutaraldehyde/0.5% paraformaldehyde in HEPES (pH 7.3) containing 0.1% picric acid. The brain was removed and post-fixed overnight at 4°C. Vibratome sections (200-mm thick) were cut in the coronal plane through the striatum and the dorsal hippocampus. A 2 × 2 $mm^2$ piece of the dorsolateral striatum (site of the major input of the corticostriatal pathway) and the CA1 subregion of the hippocampus (used as a control area to look for nonspecific effects of exercise), were dissected, washed in HEPES buffer, incubated at room temperature in the dark in aqueous 1% osmium tetroxide/1.5% potassium ferricyanide, washed in deionized water and en block stained with aqueous 0.5% uranyl acetate at room temperature for 30 min. The tissue was dehydrated, embedded in Embed 812/Spurr's (EMS; Fort Washington, PA) and sections were cut and stained. Post-embedding immunogold electron microscopy was carried out according to a modified method of Phend (Phend et al., 1992; Tillerson et al., 2003). Thin sections (light gold interface color) were cut and placed on 200-mesh nickel coated grids double coated previously with a solution from a Coat-Quick "G" pen (Kiyota International, Elk Grove, IL.), air dried for several hours, and washed for 5 min in TBS with Triton X-100 (TBST; 0.05 M Tris, pH 7.6, 0.9% NaCl, and 0.1% Triton X-100). The grids were transferred to the primary antibody solution and incubated overnight in a moist chamber. The glutamate antibody (non-affinity purified, rabbit polyclonal; Sigma, St. Louis, MO), as characterized previously by Hepler et al. (1988), was diluted 1:400,000 in TBST 7.6. Aspartate (1 mM) was added to the glutamate antibody mixture 24 hr before incubation with the thin-sectioned tissue to prevent any cross-reactivity with aspartate within the tissue. The grids were incubated for 1.5 hr at room temperature in goat anti-rabbit IgG conjugated to 10-nanometer gold (diluted 1:50 in TBST 8.2; Amersham). Photographs (10/animal) were taken randomly throughout the section containing the caudate nucleus or the CA1 region of the hippocampus (1 section/grid, one photograph per grid square) at a final magnification of 40,000× within the area of the neuropil (location of the greatest number of synapses) by an individual blinded to the particular experimental group and then captured on the computer using an AMT (2K × 2K) digital camera (Danvers, MA). The number of gold particles per nerve terminal associated with an asymmetrical (glutamate) synaptic contact and the area of the nerve terminal was determined using Image Pro Plus imaging software (Media Cybernetics, Tacoma, WA). The gold particles contacting the synaptic vesicles within the nerve terminal were counted and considered part of the vesicular or neurotransmitter pool by previously determined methods (Meshul et al., 1998; Tillerson et al., 2003). The specificity of immunolabeling for the glutamate antibody was established by incubating the antibody overnight with 3 mM glutamate. Preabsorption of the glutamate antibody resulted in a total lack of tissue labeling. The density of glutamate immunolabeling within the mitochondria associated with the presynaptic terminal was determined also as a means of investigating changes in the presynaptic metabolic pool of glutamate. The mean density of gold particles/$\mu m^2$ ± SEM) was determined within each treatment group. Stereologic analysis was not carried out for the nerve terminal glutamate immunolabeling study, because synapse density was not being determined, only the density of gold particles per identified nerve terminal making an asymmetrical synaptic contact.

### In Situ Hybridization

Brains for in situ hybridization were removed quickly and frozen in isopentane on dry ice. Sections were cut 14-μm thick on a Jung 1850 cryostat (Leica, Inc.) and thaw mounted onto poly-L-lysine-coated microscope slides, dried on a 55°C slide warmer, and fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS), pH 7.2. Sections were dehydrated in successive ethanol washes (30, 60, 80, 95, and 100%), deprotonated in triethanolamine/acetic anhydride, delipidated in chloroform, and dehydrated in ethanol. Slides containing tissue sections were exposed to hybridization buffer containing 4× standard sodium citrate (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 50% formamide, 1× Denhardt's Solution, 250 μg/ml tRNA, 500 μg/ml salmon sperm DNA, and 10% dextran sulfate with 1 × $10^6$ cpm of probe. Sections were cover-slipped and incubated overnight at 44°C. Tissue sections labeled with ribonucleotide probes were washed first in 2× SSC/50% formamide/0.1% β-mercaptoethanol at 37°C for 30 min, then 20 μg/ml RNase in 0.5 M NaCl/10 mM Tris pH7.4 at 37°C for 30 min, followed by 2× SSC/50% formamide/0.1% β-mercaptoethanol at 60°C for 1 hr, 0.1× SSC/0.1% β-mercaptoethanol at 65°C for 1 hour, and finally rinsed in ethanol before air drying. Slides were placed against high-resolution film (Hyperfilm B-max; Amersham) with radioactive standards (Amersham, Inc.). Selected slides were dipped in NTB-2 (Kodak) photographic emulsion, developed in D-19 developer and counter stained with eosin. To minimize potential sources of variation between different experiments, slides that were to be compared were processed in the same experiment using identical hybridization cocktail, probe concentration, probe preparation, wash regimen, and film exposure. The computerized image analysis program Bioquant was used to

determine the number of emulsion grains above specific anatomic regions within the substantia nigra pars compacta.

### Statistical Analysis

Linear regression was carried out to compare the rate of change in velocity and endurance of treadmill running between the two groups. Treatment groups were compared using one-way analysis of variance (ANOVA), followed by the Fisher post hoc test for comparison of multiple means for the following measures: DAT, TH, dopamine D1 and D2 receptors, and glutamate immunogold labeling. All analyses were carried out with SPSS software. Statistical significance was accepted at $P < 0.05$.

## RESULTS

### Exercise-Induced Changes in Behavior

Figure 1 outlines the four animal groups used in this study. Two groups (saline + exercise and MPTP + exercise) were subjected to treadmill exercise for 30 days and changes in running duration and velocity were measured. Duration increased over the 30-day exercise period for both groups (see Fig. 2A). Both the saline + exercise and MPTP + exercise groups were capable initially of running for a duration of 30 min on Day 1, which increased to a maximal duration of 60 min. The saline + exercise group reached maximal duration by Day 12, however, whereas the MPTP + exercise group did not reach maximal duration until Day 26. As such, we compared the rate of increase in exercise duration over the first 12 days of running. The rate of change of duration for the saline + exercise group was significantly greater than that seen in the MPTP + exercise group ($P < 0.05$).

Similar to duration, running velocity increased in both the saline + exercise and MPTP + exercise groups over the 30-day period (see Fig. 2B). The saline group ran at a velocity of 13.3 m/min at Day 1 and increased to 23.0 m/min by Day 30. The MPTP group ran at a velocity of 6.3 m/min at day 1 and increased to 21.7 m/min by day 30. There was a significant difference in velocity at Day 1 between the two groups (saline + exercise, 13.3 m/min; MPTP + exercise

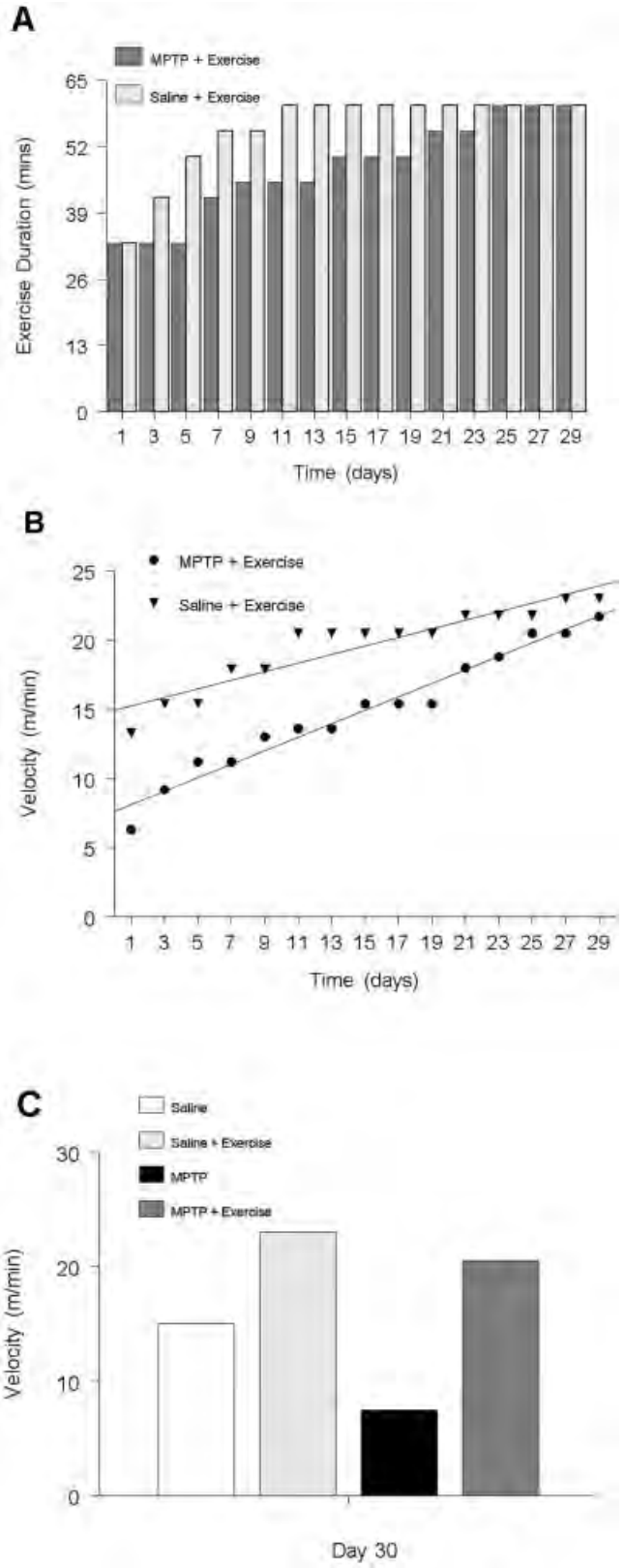


Fig. 2. Exercise-induced changes in behavior. **A:** Change in running duration over the 30-day running period for the saline + exercise group (gray bars) and MPTP + exercise group (black bars). The bars represent the performance of all 10 mice/group running at the same time. The increase over days of running reflects that all 10 mice met the criteria for increasing running duration. No statistical analysis was carried out because each bar represents all 10 mice/group as a single data point. **B:** Change in running velocity (in m/min) over the 30-day running period for the saline + exercise group (triangles) and MPTP + exercise group (circles). Symbols represent the performance of all 10 mice in each running group; increase over days of running reflects that all 10 mice met the criteria for increasing running velocity. **C:** Compares running velocity between the four groups (saline, white bar; MPTP, light gray bar; saline + exercise, black bar; and MPTP + exercise, dark gray bar) at the conclusion of the running program on Day 30. The bars represent performance of all 10 mice/group from the four groups running at the same time.

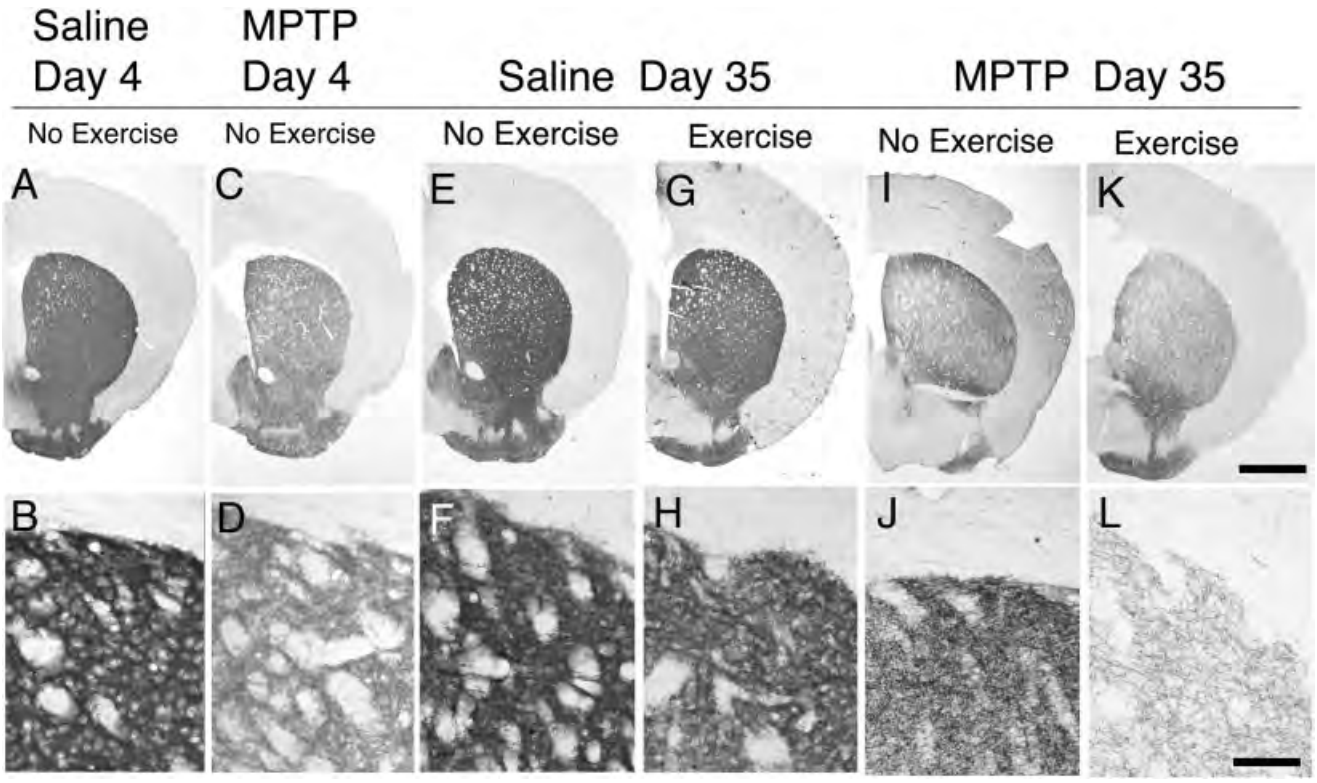


Fig. 3. Exercise-induced changes in striatal dopamine transporter immunolabeling. The relative expression of striatal dopamine transporter protein (DAT) after MPTP lesioning and between different treatment groups was determined using immunohistochemistry. The upper panels show low-magnification (10×) images of coronal sections at the level of the midstriatum stained with an antibody against DAT protein. The lower panels show high-magnification (400×) images from a region corresponding to the dorsal lateral striatum from respective sections in the upper panels. MPTP lesioning leads to a reduction in DAT-ir (comparing saline in **A** and **B** and MPTP in **C** and **D** at Day 4). MPTP + exercise showed reduced DAT-ir (**K** and **L**) compared to MPTP without exercise (**I** and **J**). Saline + exercise (**G** and **H**) showed a slight reduction in DAT-ir compared to saline alone (**E** and **F**). Scale bar + 0.5 mm (in K, for upper panels); 50 μm (in H, for lower panels).

group, 6.3 m/min; 7.0 m/min difference between groups at Day 1; $P < 0.0001$). This difference reduced to 1.3 m/min by Day 30 (Fig. 2B). The change in velocity over days of running resulted in a significantly different rate of change between the two groups ($P < 0.0001$). The MPTP + exercise group increased treadmill velocity by 5 m/min per day compared to 3.1 m/min per day for the saline + exercise group (Fig. 2B).

At the end of the 30-day running period, all animals from the four groups (saline, saline + exercise, MPTP, and MPTP + exercise) were tested on the treadmill to compare running speed capability (Fig. 2C). The MPTP and MPTP + exercise groups had a treadmill velocity of 7.5 and 21.7 m/min, respectively. Interestingly, the running velocity of the MPTP group at 35 days post-MPTP lesioning (7.5 m/min) was similar to the MPTP + exercise group at Day 1 (6.3 m/min) of their treadmill exercise program. Taken together, these findings indicate that there was no spontaneous increase in running velocity in the MPTP nonexercised group. The intensity of our training regimen was substantiated by the fact that there was an effect of exercise in the saline + exercise group compared to the saline nonexercised group. The comparison of running speed capability (Fig. 2C) at the end of the exercise program demonstrated differences in running velocity between the saline groups (saline + exercise, 23 m/min; saline nonexercised, 15 m/min).

## Exercise-Induced Changes in Striatal Dopamine Transporter Protein

Analysis of the pattern of expression of DAT protein in the midstriatum (Bregma level +1.00) in both the saline (Fig. 3A,B) and MPTP groups (Fig. 3C,D) at Day 4 showed a significant reduction in DAT-ir due to MPTP lesioning. After the exercise regimen (Day 35 post-lesioning) the saline group showed the highest degree of striatal DAT-immunoreactivity (DAT-ir) compared to that in all other groups (Fig. 3E,F). Interestingly, the saline + exercise group had reduced DAT-ir compared to the saline group, which suggests that exercise itself reduced DAT-ir (compare Fig. 3E,F with Fig. 3G,H). This reduction was shown to be significant (saline, 100.0 ± 3.1%; saline + exercise, 84.3 ± 2.8%; $P < 0.006$) (see Fig. 4). In addition, the MPTP + exercise group demonstrated sig-

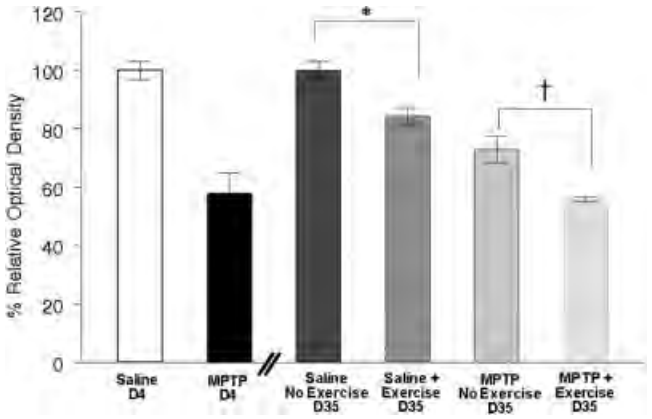


Fig. 4. Analysis of exercise-induced expression of dopamine transporter immunolabeling: The relative striatal DAT-ir was determined by measuring the relative optical density of the dorsal lateral quadrant (in at least 12 sections) correcting for staining background within sections by measuring the relative OD of the corpus callosum. This data represents relative staining for representative sections from the saline and MPTP groups at Day 4 post-MPTP lesioning and the four treatment groups collected after completion of the exercise program at post-MPTP lesioning Day 35. For comparisons, the Day 4 saline group was arbitrarily set as 100% (mean ± SEM, 100 ± 3.1%) and the relative optical densities of all other groups were normalized against it. Comparison of DAT-ir at Day 4 post-MPTP lesioning (58.0 ± 7.0%) showed a significant reduction in DAT-ir. The asterisks and cross indicate statistically significant differences between the saline and saline + exercise and the MPTP and MPTP + exercise groups, respectively. This reduction was significant (saline, 100.0 ± 3.1%; saline + exercise, 84.3 ± 2.8%; $P < 0.006$). In addition, the MPTP + exercise group demonstrated significantly reduced DAT-ir compared to the MPTP group (MPTP, 73.3 ± 4.5%; MPTP + exercise, 56.0 ± 0.7%; $P < 0.028$).

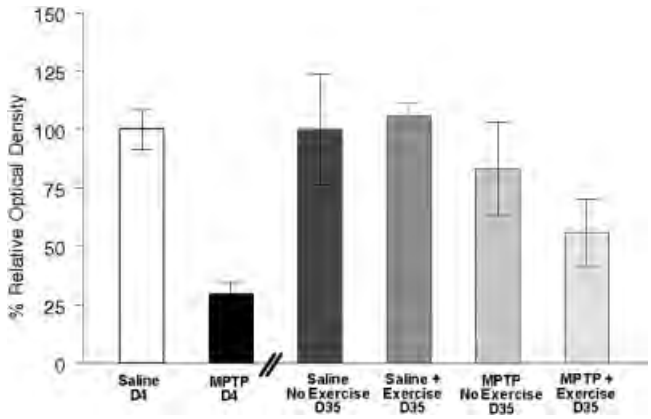


Fig. 5. Analysis of the relative striatal TH-protein expression by Western immunoblotting. The relative optical density measurement of the saline group at Day 4 post-lesioning was set arbitrarily at 100 ± 3.1% for comparison between different treatment groups. Analysis of TH-protein levels at 4 days after MPTP lesioning showed a significant reduction (30.0 ± 4.5%) compared to that in saline controls. There were no significant differences between groups in TH-ir at 35 days post-MPTP lesioning (saline, 100.0 ± 23.8%; saline + exercise, 105.8 ± 5.7%; MPTP, 83.3 ± 20.0%; MPTP + exercise, 56.0 ± 14.3%; $P + 3.72$). These data, however, indicate a small, nonsignificant reduction in the relative density of TH-ir in the MPTP groups compared to that in the saline groups. In addition, TH-ir was reduced slightly in the MPTP + exercise group compared to MPTP alone.

nificantly reduced DAT-ir compared to the MPTP group (MPTP, 73.3 ± 4.5%; MPTP + exercise, 56.0 ± 6.3%; $P < 0.028$) (see Fig. 4 and compare Fig. 3I,J with Fig. 3K,L).

## Exercise-Induced Changes in Tyrosine Hydroxylase Striatal Protein

Western immunoblotting with an antibody recognizing TH protein was carried out on striatal tissue at post-lesioning Day 4 and at the end of the exercise program (35 days after MPTP lesioning) (Fig. 5). Comparison of the Day 4 saline with the Day 4 MPTP lesioning showed a significant reduction in striatal TH protein due to MPTP lesioning (saline, 100 ± 3.0% compared to MPTP, 30.0 ± 4.5%). Comparison of the level of TH protein at the end of the running regimen (at Day 35) showed that there was no significant difference in TH immunoreactivity between any of the groups (saline, 100.0 ± 23.8%; saline + exercise, 105.8 ± 5.7%; MPTP, 83.3 ± 20.0%; MPTP + exercise, 56.0 ± 14.3%, $P + 3.72$). Although not significant (35 days after MPTP lesioning), MPTP groups (MPTP and MPTP + exercise) showed a slight reduction in TH immunoreactivity compared to the saline groups. In addition, TH immunoreactivity was reduced slightly in the MPTP + exercise group compared to that in the MPTP group.

## Exercise-Induced Changes in Dopamine D1 and D2 Receptors

In situ hybridization histochemistry with probes recognizing either the D1 or D2 subtype of dopamine receptor was carried out on dorsal striatal tissue of mice from all groups. Compared to the saline group, expression of D1 mRNA was reduced as a result of exercise, MPTP lesioning, or both (saline, 100.0 ± 6.9%; saline + exercise, 51.9 ± 3.9%; MPTP, 50.1 ± 5.9%; MPTP + exercise, 48.6 ± 4.2%; $P < 0.0001$) (Fig. 6A). Comparison of the MPTP with the MPTP + exercise group showed no difference in the level of dopamine D1 mRNA expression. The expression of dopamine D2 mRNA was also reduced as a result of either exercise (saline + exercise) or MPTP lesioning (MPTP group) compared to that in the saline group (saline, 100.0 ± 7.6%; saline + exercise, 58.5 ± 6.4%; MPTP, 50.1 ± 5.7%; $P < 0.002$) (Fig. 6B). The combination of MPTP lesioning and exercise (MPTP + exercise group), however, resulted in no difference in dopamine D2 mRNA expression when compared to that in the saline group, but was increased significantly when compared to that in the MPTP group (MPTP + exercise, 95.9 ± 9.4%; $P < 0.005$).

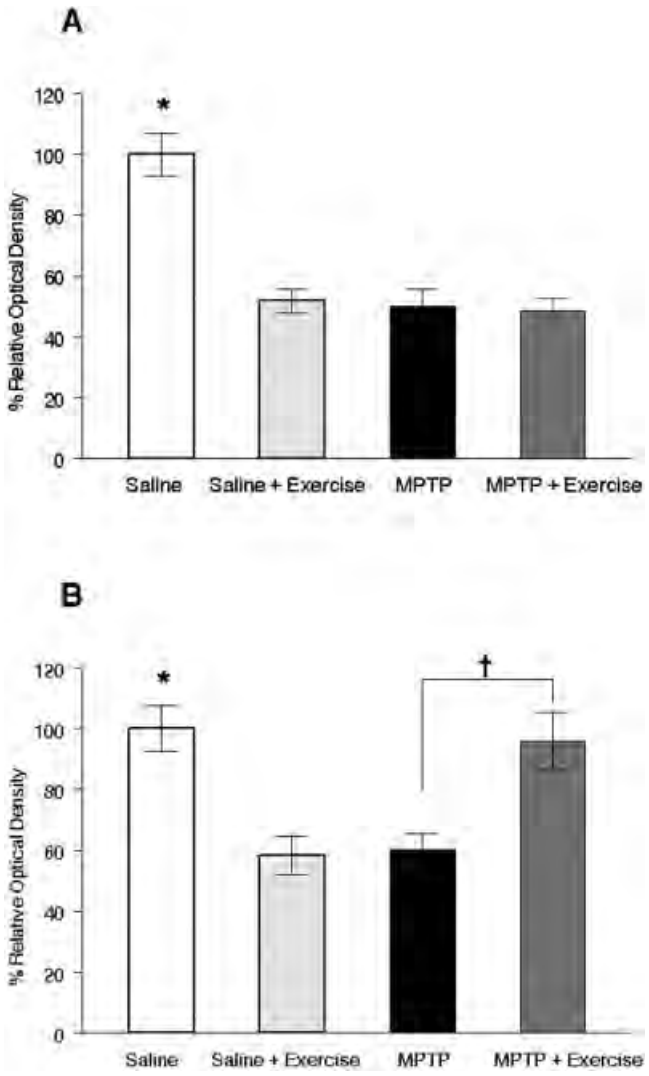


Fig. 6. Analysis of the relative striatal dopamine D1 and D2 receptor mRNA using in situ hybridization histochemistry. The relative optical density of autoradiographic grains above the dorsal striatum were determined from at least three mice from each treatment group using at least 12 sections/mouse. For comparison, the saline group was set arbitrarily at 100% and all other groups normalized against it for both dopamine receptors D1 and D2 mRNA. **A:** Compared to the saline group, expression of D1 mRNA was reduced significantly with exercise and MPTP lesioning (saline, 100.0 ± 6.9%; saline + exercise, 51.9 ± 3.9%; MPTP, 50.1 ± 5.7%; MPTP + exercise, 48.6 ± 4.2%; $P + 0.0001$). **B:** The expression of dopamine D2 mRNA was reduced as a result of either exercise (saline + exercise) or MPTP lesioning (MPTP group) compared to that in saline group (saline, 100.0 ± 7.6%; saline + exercise, 58.5 ± 6.4%; MPTP, 60.3 ± 5.3%; $P < 0.002$). The expression of dopamine D2 mRNA was increased significantly in the MPTP + exercise group when compared to that in the MPTP group (MPTP + exercise, 95.9 ± 9.4%; $P < 0.005$).

### Exercise-Induced Changes in Nerve Terminal Glutamate Immunolabeling

Immunogold electron microscopy was used to determine the density of nerve terminal glutamate immunolabeling in mice from all groups at completion of the exercise program. Figure 7 shows representative images of asymmetrical (excitatory) synaptic contacts labeled for the neurotransmitter glutamate in mice from the saline group, saline + exercise group, MPTP group, and the MPTP + exercise group (Fig. 7A–D, respectively). There was a significant increase in the density of nerve terminal glutamate immunolabeling in the MPTP group compared to saline, (values are mean number of gold particles/$\mu m^2$ ± SEM: saline, 85.9 ± 3.6; MPTP, 135.3 ± 12.4; $P < 0.05$) (Fig. 8). Additionally, there was a significant decrease in the density of nerve terminal glutamate immunolabeling in the MPTP + exercise group compared to the MPTP group (MPTP, 135.3 ± 12.4; MPTP + exercise, 105.3 ± 4.5; $P < 0.05$). This decrease reached levels that were similar to the saline groups (mean number of gold particles/$\mu m^2$ ± SEM: saline, 85.9 ± 3.6; saline + exercise, 102.6 ± 3.7; MPTP, 135.3 ± 12.4; MPTP + exercise, 105.3 ± 4.5).

To determine the specificity of the change in the density of glutamate immunolabeling within the nerve terminal, the density of labeling within the presynaptic mitochondrial pool was quantified. There was no difference between any of the experimental groups (data not shown). Additionally, there were no changes in nerve terminal area between groups (data not shown).

Glutamate immunolabeling of the CA1 in the hippocampus was analyzed to confirm that the observed differences between groups in glutamate immunolabeling were specific to the striatum. This brain area was chosen because it also receives a significant glutamatergic input and is associated with spatial learning as opposed to the dorsolateral striatum that is associated primarily with motor function. There were no differences in the density of nerve terminal glutamate immunolabeling between any of the groups as shown in Figure 8B (values are mean number of gold particles/$\mu m^2$ ± SEM; saline, 100.0 ± 5.7; saline + exercise, 101.7 ± 7.9; MPTP, 88.4 ± 4.6; MPTP + exercise, 103.2 ± 6.6; $P < 0.49$).

## DISCUSSION

The purpose of this study was to examine the effect of exercise on restoration of surviving dopaminergic neurons after completion of MPTP-induced cell death. The MPTP lesioning regimen used in our studies involves a series of four injections of 20 mg/kg (free-base) leading to a 60–70% loss of nigrostriatal dopaminergic neurons and a 90–95% depletion of striatal dopamine (Jackson-Lewis et al., 1995). Using this regimen, nigrostriatal cell loss is complete by Day 3 after MPTP administration and shows no further decline 30 days post-lesioning as determined by either unbiased stereologic counting techniques (Petzinger et al., in preparation) or section-sampling techniques (Jackson-Lewis et al., 1995). Despite the extent of cell loss, MPTP-lesioned mice display robust and reproducible return of striatal function 2–3 months after injury (Ricaurte et al., 1986; Jakowec et al., 2003). The levels of TH and DAT immunoreactivity (TH-ir and DAT-ir) are de-

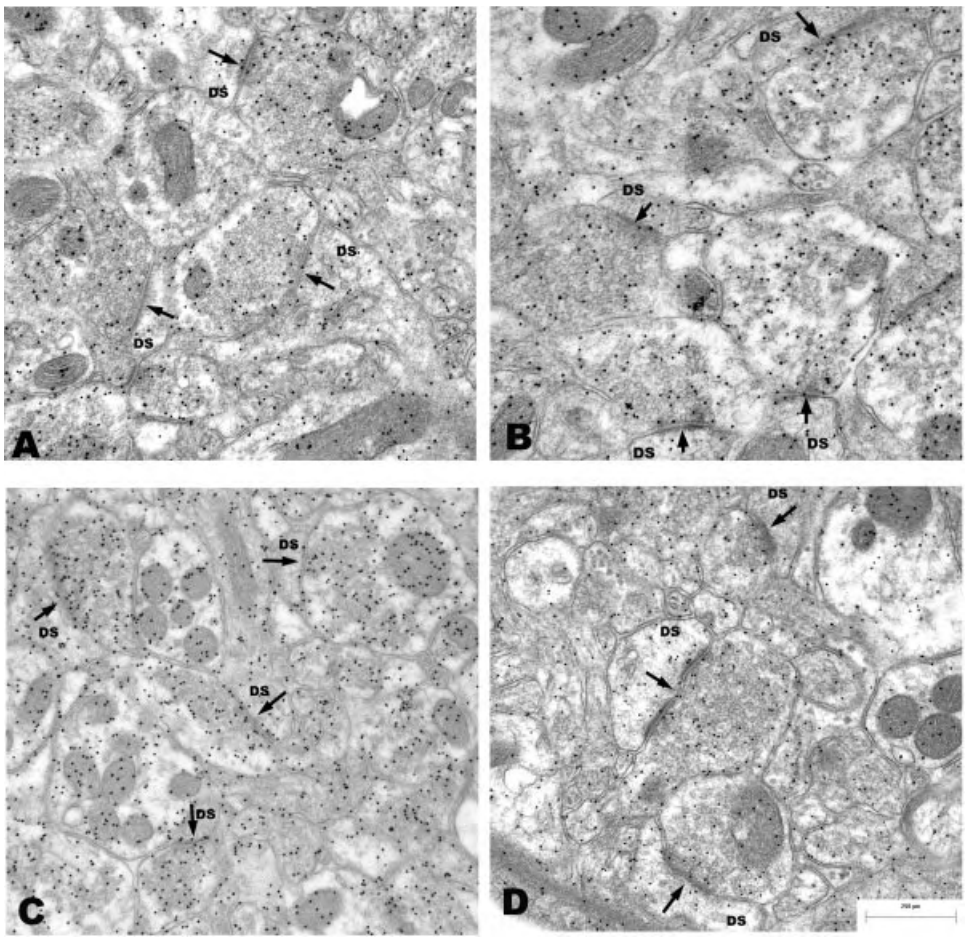


Fig. 7. Electron photomicrographs using the immunogold technique to localize an antibody against the neurotransmitter, glutamate, within the dorsolateral striatum. **A:** Saline group. Three nerve terminals are seen making an asymmetrical synaptic contact (arrows) with an underlying dendritic spine (DS). Within the nerve terminal are numerous 10-nm gold particles, indicating the location of the antibody. These gold particles are found overlying the round synaptic vesicles. **B:** Saline group that was exercised for 30 days, starting 4 days after the injection of saline. Note that the density of nerve terminal glutamate immunolabeling seems similar to that seen in the saline-treated group in A. **C:** MPTP-treated group was given an acute injection of the toxin (20 mg/kg × 4 injections every 2 hr) and then perfused with fixative 34 days later. Note the increase in the density of immunogold particles in all three nerve terminals compared to that observed in the saline group shown in A. **D:** MPTP-treated group that was exercised for 30 days, starting 4 days after the acute toxin treatment. Note that the density of glutamate immunogold labeling is similar to that seen in the saline-treated group in A. Scale bar + 0.25 μm.

creased to 30% of pre-MPTP-lesioned levels by Day 7 and return to 50–60% or more of pre-MPTP-lesioned levels within 30–60 days (Jakowec et al., 2003, 2004). These changes are part of molecular alterations underlying intrinsic neuroplasticity in this model (Jakowec et al., 2004) and are represented in this study by the MPTP nonexercised group. The saline group in this study served two purposes: (1) to show that the intensity of the exercise regimen was sufficient to induce a behavioral effect in non-lesioned controls; and (2) to compare the effect of exercise on the noninjured and injured brain.

Using a high-intensity (high velocity and duration) treadmill exercise paradigm in the MPTP-lesioned mouse, we have shown that exercise leads to behavioral recovery, specifically amelioration of initial deficits in running speed and duration compared to nonexercised MPTP-lesioned animals. Importantly, as would be expected by an effective training paradigm, the non-lesioned animals that exercised demonstrated enhanced performance compared to their non-lesioned, nonexercised counterparts. Specifically these behavioral differences between exercised and non-exercised mice consisted of the capability of the exercised saline mice to run at higher velocities after a 30-day treadmill-training program. We showed that MPTP-lesioned and non-lesioned mice could be forced to run at progressively faster speeds and longer durations and learn to associate a sensory stimulus with a behavioral response (i.e., maintaining a specific position on the treadmill). Over time, sensory feedback was no longer necessary for the animals to maintain a forward position, indicating that learning had occurred.

Although the MPTP-lesioned mouse displays subtle motor behavioral deficits that may not be evident under normal caging conditions, motor deficits become evident under specific task or environmental manipulations (Sedelis et al., 2001; Tillerson et al., 2002). Bradykinesia (slowness of movement) and fatigability (decreased endurance) were two behavioral deficits we observed initially in treadmill-exercised mice after MPTP lesioning. MPTP-lesioned mice were not capable of running at the same treadmill velocity as the saline + exercise mice in the first 18 days of running. By the end of the 30-day exercise program, the MPTP + exercise mice were running at a velocity near that of the saline + exercise group and greater than that of the saline (nonexercised) group (see Fig. 2C). The rate of change of velocity was greater in the

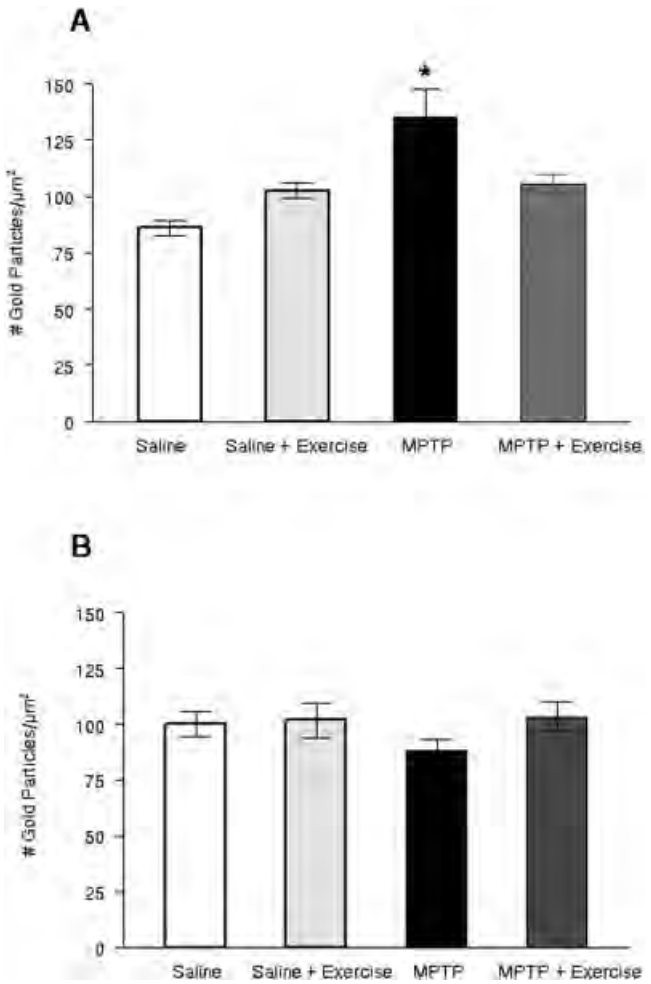


Fig. 8. Quantification of immunogold electron microscopy results. **A:** There was a significant increase in the density of synaptic glutamate immunolabeling within striatal nerve terminals making asymmetrical synaptic contacts after the acute administration of MPTP compared to the other treatment groups (mean number of gold particles/$\mu m^2$ $\pm$ SEM: saline, 85.9 $\pm$ 3.6; saline + exercise, 102.6 $\pm$ 3.7; MPTP, 135.3 $\pm$ 12.4; MPTP + exercise, 105.3 $\pm$ 4.5; $P < 0.05$). **B:** Using quantitative immunogold electron microscopy, there was no difference in the density of glutamate immunolabeling within nerve terminals making an asymmetrical synaptic contact within the CA1 region of the hippocampus between any of the treatment groups. $*P < 0.05$ compared to all the other groups as determined by the Fisher post-hoc test for comparison of multiple means.

injured animals compared to their nonlesioned exercised counterparts. This result is in accordance with exercise studies in cortically injured animals in which the effect of exercise is greater after injury. Injury may prime the system for adaptation perhaps through the induction of neurotrophic factors including brain-derived neurotrophic factor (BDNF) (Cotman and Berchtold, 2002; Gomez-Pinilla et al., 2002; Cohen et al., 2003).

Tillerson et al. (2003) also reported behavioral improvement after treadmill exercise in two rodent models of basal ganglia injury (the 6-OHDA rat and MPTP-lesioned mouse), which was associated with attenuation of dopamine loss (Tillerson et al., 2003). The investigators concluded that exercise might work largely through neuroprotective mechanisms because exercise was started within 12 hr of lesioning, and MPTP and 6-OHDA may take several days to complete cell death (Sauer and Oertel, 1994; Jackson-Lewis et al., 1995). Unlike the Tillerson et al. (2003) study, the focus of our study was to address the effect of high-intensity treadmill exercise on the neurorestoration of surviving neurons after MPTP lesioning. This was accomplished by: (1) initiating exercise 4 days after lesioning, a time period well after cell death is completed in this model; (2) continuing exercise over a 30-day period; and (3) progressively increasing treadmill velocity and duration over that period. Two additional differences between Tillerson et al. (2003) and the present study were the exercise parameters and the age of the animals. Our exercise parameters on young mice were of higher velocity, duration, and frequency and showed an effect on the saline + exercise group that was not seen in the Tillerson et al. (2003) study.

In addition to a behavioral effect, exercise resulted in decreased DAT-ir compared to the nonexercised groups. The effect of exercise on DAT-ir was even greater in the MPTP group. In the basal ganglia, the biosynthesis of dopamine is dependent on the enzyme tyrosine hydroxylase (TH) and the primary mechanism of clearance of dopamine from the extracellular space is through the dopamine transporter (DAT) (Gainetdinov et al., 2002; Mortensen and Amara, 2003). Several mechanisms have been shown to regulate DAT activity including: (1) gene and protein expression of transporter number; (2) phosphorylation activated through glutamate receptors such as the mGluR5 metabotropic receptor; and (3) internalization within endosomes mediated by dopamine (Perrone-Calano et al., 1996; Page et al., 2001). Alterations in DAT activity can influence the synaptic occupancy of dopamine. An intervention (such as exercise) that downregulates DAT-ir expression may therefore lead to behavioral improvement by increasing synaptic occupancy of dopamine. The downregulation of DAT-ir in our exercised animals could account for the superior running capabilities of both the MPTP and saline groups compared to the nonexercised groups. This interpretation is consistent with the findings of Meeusen et al. (1997) and others that report increased extracellular levels of dopamine with exercise. An alternative explanation for reduced DAT-ir in our exercised animals is the loss of nigrostriatal terminals where DAT normally resides. This does not seem likely because TH-ir, another marker of nigrostriatal terminal integrity, was not altered significantly and because exercised animals had superior running capability compared to their nonexercised counterparts, which would not be expected if cell death were ongoing. Studies are underway currently to investigate the possibility of cell death and terminal loss

including stereologic cell counting of substantia nigra pars compacta neurons and fiber density, respectively.

In saline animals, exercise suppressed dopamine D1 and D2 receptor mRNA levels. In the MPTP group, exercise seemed to have no effect on D1 but increased D2 mRNA levels. Of the dopamine receptor superfamily, D1 and D2 subtypes are the most prevalent in the striatum (REF). Activation of these receptors by dopamine leads to the release of neuropeptides from medium spiny neurons. Medium spiny neurons with D1 receptors express the neuropeptide preprotachykinin (PPT) and medium spiny neurons with D2 receptors express the neuropeptide preproenkephalin (PPE) (Gerfen, 2000). In the normal brain, D1 and D2 act synergistically and activation of both is required to elicit a motor response (Gerfen et al., 1995). In the lesioned basal ganglia, this synergy is lost and activation of either D1 or D2 may elicit a motor response. In addition, D2 activation alone in the injured state seems to elicit a more robust motor response that may be attributed to its heightened sensitivity after lesioning (LaHoste and Marshall, 1993). In our study, exercise seems to have a similar effect on both receptor subtypes in the saline animals that may reflect the synergy normally seen in the uninjured basal ganglia. This synergy, however, is lost in injury and affects each receptor subtype differently. The loss of synergy between the dopamine D1 and D2 receptors due to injury by MPTP is revealed in the context of exercise. One possible mechanism to explain the differential effect of exercise and injury on the dopamine receptors subtypes D1 and D2 may be through the action of glutamate. Glutamate has been shown to influence subtype-specific regulation of the dopamine receptors (see discussion below). The combination of the upregulation of D2 mRNA (leading to increased motor activity) along with the downregulation in DAT (leading to increased synaptic occupancy of dopamine) may explain the behavioral improvement seen in the MPTP + exercise mice. Studies are underway currently to localize the dopamine D2 receptor changes with exercise and to determine if D1 or D2 receptor antagonists or agonists affect the behavioral benefits of exercise.

Glutamate is the major excitatory neurotransmitter in the brain and plays an important role in motor behavior (Starr, 1995). Glutamate is stored within nerve terminals and upon release binds to a superfamily of receptors including the *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA), and kainic acid (KA) subtypes. In the striatum, the primary glutamatergic pathway is the corticostriatal input to the medium spiny neurons (Starr, 1995). One method for examining changes in glutamatergic neurotransmission is to measure alterations in glutamate storage within striatal nerve terminals using immunogold electron microscopy. We observed an increase in the density of nerve terminal glutamate immunolabeling in animals after MPTP lesioning. This increase was reversed by exercise to levels seen in the saline control groups. Additionally, this effect was specific to the lesioned dorsal-lateral striatum (an area associated primarily with motor function) because there was no alteration in CA1 glutamate terminals originating from either the Schaffer collaterals or from the contralateral hippocampus (an area associated primarily with learning and memory). In comparison to the MPTP-lesioned animals, no significant change in immunogold labeling was observed between the saline and saline + exercise groups. A change in glutamate terminal storage in the nonlesioned brain may require a higher intensity of exercise then used in the present studies (Meeusen et al., 1997).

Although glutamate levels were not measured in this study, Meshul et al. (2000) has shown an inverse relationship between terminal glutamate immunogold labeling and levels of glutamate within the synapse. An increase in the density of nerve terminal glutamate immunolabeling (as is seen with MPTP lesioning) may therefore reflect a decrease in the extracellular levels of striatal glutamate. Consequently, one hypothesis with respect to our results is that an effect of exercise in the MPTP-lesioned brain may be to increase the release of glutamate at the synapse, which that may alter dopamine receptor subtype expression or medium spiny neuron peptide expression (Cepeda et al., 1993; Cepeda and Levine, 1998; Liste et al., 1999). Using microdialysis in the 6-OHDA rat, Meeusen et al. (1997) showed an increase in extracellular glutamate with exercise (Meeusen et al., 1997; Bland et al., 1999).

Studies have shown that there are close interactions between glutamate and dopamine neurotransmission in mediating motor control (Starr and Starr, 1994; Starr, 1995; Starr et al., 1997). The striatal medium spiny neuron is thought to be the site for integrating these interactions. Exercise may either directly affect the medium spiny neuron or indirectly influence its afferents. For example, expression of the immediate early gene cFos (a marker of cell activation) and the neuropeptides preprotachykinin and preproenkephalin in medium spiny neurons are altered by exercise (Cepeda et al., 1993; Liste et al., 1999). Altered expression of these markers in medium spiny neuron activity can be blocked by either glutamate or dopamine receptor antagonists or through denervation. The present study has shown changes in both glutamate and dopamine systems. We do not yet know, however, if glutamate and dopamine changes are dependent or mutually exclusive of each other. To test the degree of dopamine–glutamate interactions with exercise, we are conducting additional studies with exercise in MPTP and saline mice administered either glutamate or dopamine antagonists. If the suppression of DAT-ir seen in our studies can be blocked by administration of a glutamate antagonist during exercise, it would support the hypothesis that glutamatergic neurotransmission is important in regulating exercise-induced changes in dopamine function. Furthermore, dopamine receptor-specific agonists and antagonists targeting either D1 or D2 will test whether the alterations in glutamate immunolabeling seen in our studies are dependent on dopamine neurotransmission.

In conclusion, exercise may be both neuroprotective and neurorestorative in the injured basal ganglia. It has been shown previously that initiating exercise at or during the time of neurotoxin-induced cell death is neuroprotective by attenuating striatal dopamine loss (Cohen et al., 2003; Tillerson et al., 2003). Our studies show that a high-intensity treadmill exercise paradigm initiated after the period of neurotoxin-induced cell death is neurorestorative as demonstrated through its beneficial effect on motor behavior. Alterations in both dopaminergic and glutamatergic neurotransmission in response to exercise may underlie the molecular mechanisms of this effect. The potential impact of this study is that exercise may not only play a role in prevention of Parkinson's disease but in restoring function in individuals who have been diagnosed with Parkinson's disease.

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SYNAPSE 58:200–207 (2005)

# High and Low Responders to Novelty Show Differential Effects in Striatal Glutamate

SAATE S. SHAKIL,[1] HALEY K. HOLMER,[1] CYNTHIA MOORE,[1] AVERY T. ABERNATHY,[2] MICHAEL W. JAKOWEC,[2] GISELLE M. PETZINGER,[2,3] AND CHARLES K. MESHUL[1,4]*
[1]*Research Services, Neurocytology Lab, V.A. Medical Center, Portland, OR 97239*
[2]*Department of Cell and Neurobiology, The George and MaryLou Boone Parkinson's Disease and Movement Disorders Research Center, Los Angeles, CA 90033*
[3]*Department of Neurology, University of Southern California, Los Angeles, CA 90033*
[4]*Department of Behavioral Neuroscience, Oregon Health & Science University, Portland, OR 97239*

*KEY WORDS* in vivo microdialysis; striatum; glutamate; dopamine; electron microscopy; individual differences

*ABSTRACT* The goal of this study was to determine whether there was a difference in glutamate within the dorsolateral striatum in mice exhibiting either a high (HR) or low (LR) locomotor response to a novel environment. The number of line crossings over a 30-min-period when the mice were placed in a novel environment was determined, and those mice for which the values were above the mean were in the HR group and those with the values below the mean were in the LR group. In vivo microdialysis was carried out to determine the basal extracellular level of striatal glutamate, and the contralateral striatum was taken to measure the density of glutamate immunolabeling within nerve terminals making an asymmetrical (excitatory) synaptic contact using quantitative immuno-gold electron microscopy. There was a statistically significant difference (35%) in the basal extracellular level of striatal glutamate between the HR and LR groups, with the HR group having a lower level, compared with that of the LR group. There was a 25% difference in the density of nerve terminal glutamate immuno-gold labeling associated with the synaptic vesicle pool in the HR, compared with that in the LR group, but this difference was not statistically significant. There was no change in the basal extracellular level of striatal dopamine between the two groups, but there was a statistically significant difference (73%) in the basal turnover ratio of striatal dopamine and its metabolites in the HR, compared with that in the LR group. The data suggests that the difference in extracellular striatal glutamate between the HR and LR groups is not due to an alteration in basal extracellular dopamine but could be due to an increase in dopamine turnover. **Synapse 58:200–207, 2005.** 

## INTRODUCTION

It is well established that naïve rodents show differences in locomotor responses when exposed to a novel environment. When placed in a novel context, some rodents show a very high level of motor activity (termed high responders or HR) and others show a much lower level of activity (termed low responders or LR). In rats, the HR group shows a greater tendency to acquire and maintain psychostimulant self-administration (Grimm and See, 1997; Piazza et al., 1989; Pierre and Vezina, 1997). In addition, they also show behavioral sensitization more readily, compared with that of the LR group (Hooks et al., 1991, 1992; Pierre and Vezina, 1997).

In terms of brain neurochemistry, it has been reported that the HR group shows increased drug-stimulated dopamine levels in the striatum and nucleus accumbens, compared with those in the LR group (Bradberry et al., 1991; Hooks et al., 1991, 1992; Piazza et al., 1991; Rouge-Pont et al., 1993, 1998). To determine a possible mechanism behind these changes in dopamine, Marinelli and White (2000) measured the firing frequency of dopamine neurons within both

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*Correspondence to: Charles K. Meshul, Ph.D., Neurocytology Lab (RD-29), V.A. Medical Center, 3710 S.W. Veterans Hospital Road, Portland, OR 97239. E-mail: meshulc@ohsu.edu

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the ventral tegmental area and the substantia nigra pars compacta. Not only did the HR group self-administer more cocaine than the LR group does, but the basal firing rate and bursting activity of dopamine neurons within these midbrain areas was significantly higher in the HR vs LR groups. This possible change in activity of the dopamine neurons may be the mechanism behind the alterations in dopamine levels within the striatum and nucleus accumbens.

We have reported alterations in glutamate within both the dorsolateral striatum and nucleus accumbens following administration of psychostimulants such as cocaine and methamphetamine (Burrows and Meshul, 1997; Kozell and Meshul, 2001, 2003, 2004; McKee and Meshul, 2005). Although treatment with both these drugs of abuse result in an increase in extracellular dopamine levels (Abekawa et al., 1994; Church et al., 1987; Hurd and Ungerstedt, 1989; O'Dell et al., 1994), we have reported an increase in the extracellular level of striatal glutamate following a single dose of cocaine (McKee and Meshul, 2005) and a decrease in the density of glutamate immunolabeling within nerve terminals making an asymmetrical synaptic contact within the striatum and nucleus accumbens following either methamphetamine or cocaine, respectively (Burrows and Meshul, 1997; Kozell and Meshul, 2001). Indeed, the dorsolateral striatum has been implicated in a variety of cocaine-related behaviors including locomotion, stereotypy, and behavioral sensitization (Canales and Graybiel, 2000; Karler and Calder, 1992; Karler et al., 1995; Robinson and Becker, 1986). It is known that glutamate antagonists block acute cocaine-stimulated behaviors, suggesting not only the importance of the striatum but also of glutamate in terms of the effects of cocaine (Karler and Calder, 1992; Mao and Wang, 2000).

Although controversial, it has been reported, using electron microscopic immunolabeling, that there are dopamine D2 receptors located within nerve terminals making an asymmetrical (excitatory) contact onto dendritic spines (Sesack et al., 1994; Wang and Pickel, 2002; Hersh et al., 1995). Only about 25% of the terminals within the striatum contain these presynaptic dopamine D2 receptors (Wang and Pickel, 2002). In addition, there is other neurochemical evidence for the control of glutamate release by dopamine agonists (Bamford et al., 2004; Calabresi et al., 1992; Godukhin et al., 1984; Maura et al., 1988; Mitchell and Doggett, 1980; Rowlands and Roberts, 1980; Yamamoto and Davy, 1992). We have reported that in mice that are deficient in striatal levels of dopamine, there is a significant decrease in the release of glutamate following application of the dopamine agonist, quinpirole, compared with that of the wild-type striatum (Bamford et al., 2004). This suggests that presynaptic dopamine receptors located on glutamate nerve terminals play an important role in terms of controlling the release of glutamate.

Since the activity of dopamine neurons within the substantia nigra pars compacta of HR rodents is greater, compared with that of the LR group (Marinelli and White, 2000), we would predict that basal levels of striatal glutamate would also be decreased. Therefore, the goal of this study was to establish whether there was a difference in striatal glutamate in HR vs LR mice (C57BL/6J), using both in vivo microdialysis and quantitative immuno-gold electron microscopy, and to also determine whether there were changes in extracellular dopamine levels and turnover between the two groups of mice.

## MATERIALS AND METHODS

### Animals

Male, C57BL/6J mice ($N = 5$–6 for each group; 20–25 g, 10-weeks–old; Jackson Labs, Bar Harbor, ME) were maintained on a 12 h light/dark cycle with continuous access to food and water. All animal experiments were carried out in accordance with the National Institute of Health guide for the care and use of laboratory animals (NIH Publications No. 80–23, revised 1978), and all procedures were approved by the Portland VA Medical Center Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

### Behavioral testing: locomotor response to a novel environment

Mice were observed for locomotor response to a novel environment over a period of 30 min (between 8:00 AM and 12:00 PM). Each mouse was observed individually in a shoe box-type cage (26 cm × 26 cm × 13 cm). The cage was divided into 16 equal sections using white masking tape. A mouse passing across one of these lines was defined as a line crossing. An overall mean was obtained from the line crossings of all 11 mice; those mice that had values above the mean were placed in the high responders (HRs) group and those that had values below the mean were placed in the low responders (LRs) group.

### In vivo microdialysis

Under anesthesia (1 ml/kg of 2.5% ketamine, 1% xylazine, and 0.5% acepromazine in normal saline), mice were placed in a stereotaxic apparatus (Cartesian Research, OR) and implanted with stainless steel guide cannula (8 mm long, 21 gauge), lowered 1 mm from the surface on the left side of the skull just above the corpus callosum. The coordinates relative to bregma were 2.0 mm medial/lateral, +1.6 mm anterior/posterior, and 1.0 mm dorsal/ventral. The guide cannula was held in a fixed position by one stainless steel screw attached to the skull on the right

side and encompassed by dental cement. The animals were allowed to recover for one week before the start of the microdialysis experiment.

Dialysis probes were prepared as described by Robinson and Wishaw (1988), with modifications (Meshul et al., 1999). A day before the start of the actual dialysis procedure, the probe was lowered into the guide cannula with the entire length of the dialysis probe (1 mm cellulose tip) in the dorsolateral striatum. The probe was secured to the guide cannula with epoxy. The artificial cerebrospinal fluid (aCSF; 140 mM NaCl, 3.4 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.4 mM $NaH_2PO_4$, and 4.85 mM $NaHPO_4$, pH 7.4) flowed through the probe overnight at a rate of 0.2 μl/min. The following morning, the pump speed was increased to 2.0 μl/min for 30 min before the start of collecting samples. Six samples were then collected at each 15-min interval. At the conclusion of the experiment, the animals were perfused with glutaraldehyde fixative, vibratome sections (100 μm) cut, stained with thionin, and the site of the probe placement was verified histologically from the left side of the brain. Only animals with correctly placed probes were included in the statistical analysis of microdialysis data. The right side of the striatum was used for electron microscopic analysis (see below).

Glutamate concentration in dialysate samples was determined using a Hewlett Packard high-performance liquid chromatography (HPLC) 1090 interfaced with a Hewlett Packard 1046A Programmable Fluorescence Detector. Dialysates were derivatized with *o*-phthalaldehyde (OPA) and chromatographed according to a modification of the method of Schuster (1988), as previously reported (Meshul et al., 1999, 2002). Dialysates were derivatized by adding 1 μl of sample, 5 μl of borate buffer (pH 10.4), and 1 μl of OPA. The reaction mixture was injected into a reverse phase C18 column (HP # 79916AA), and OPA derivatives were separated using a linear gradient. Solvent A contained 0.018% (v/v) TEA, 0.3% (v/v) tetrahydrofuran, and 20 mM sodium acetate buffer, pH 7.2. Solvent B contained 40% (v/v) acetonitrile, 40% (v/v) methanol, and 20% (v/v) 100 mM sodium acetate, pH 7.4. The OPA derivatives of glutamate were detected using an excitation wavelength of 340 nm and an emission wavelength of 450 nm. Assay sensitivity was in the subpicomole range.

Dopamine and metabolite levels within the dialysate samples were determined according to the method of Kilpatrick et al. (1986). They were assayed by HPLC with electrochemical detection and injected with an ESA autosampler. Dopamine and metabolites were separated by a 150 × 3.2 mm reverse phase 3-μm diameter C-18 column (ESA, Chelmsford, MA) regulated at 28°C. The mobile phase MD-TM (from ESA) consisted of acetylnitrile in phosphate buffer, and an ion-paring agent delivered at a rate of 0.6 ml/minutes. The electrochemical detector was an ESA model Coularray 5600A with a 4-channel analytical cell with 3 set potentials at −100 mV, 50 mV, and 220 mV. The HPLC was integrated with a Dell BX-280 computer with analytical programs including ESA Coularray for Windows software and the statistics package InStat (San Diego, CA). The amount of dopamine and its metabolites, dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA), in each sample was calculated from individual standard curves.

For both the glutamate and dopamine/dopamine metabolite dialysate analysis, the 6 baseline data points were averaged at each time point, and then a grand mean was determined for the baseline. The values are expressed as the mean ± SEM in pmole/μl extracellular striatal glutamate or ng/ml extracellular dopamine, DOPAC, or HVA within the dialysate sample. The turnover ratio for dopamine for each of the animals was calculated as follows: (DOPAC + HVA)/dopamine and a grand mean (±SEM) was calculated for each of the groups. The mean probe recovery was 10.4 ± 1.2%. All the data between groups was analyzed using the Student's *t*-test.

We are well aware of the controversy regarding whether extracellular glutamate is derived from the calcium-dependent neuronal vesicular pool, the calcium independent, but neuronal, glutamate/cystine antiporter, or the nonneuronal (glial) pool (Baker et al., 2002; Timmerman and Westerink, 1997). We and others have reported that about 30% of extracellular glutamate is calcium dependent (Baker et al., 2002; Meshul et al., 2002; Wolf et al., 2000; Xue et al., 1996), and that over 60% of the $K^+$-depolarized extracellular level of glutamate is calcium dependent (Meshul et al., 1999), suggesting a role for the synaptic vesicle pool within the nerve terminal. Replacement of calcium with the divalent chelating agent, EGTA, and increasing the aCSF concentration of magnesium resulted in a significant decrease in the basal level of glutamate (Meshul et al., 2002), suggesting that a significant proportion of the resting level of striatal glutamate is of neuronal origin and not glial origin. It has also been reported that 60% of the basal extracellular level of glutamate is due to exchange with the glutamate/cystine antiporter, which has been shown to be calcium insensitive and most likely of cytoplasmic, but still of neuronal origin (Baker et al., 2002).

### Immunogold electron microscopy

Following the end of dialysate sample collection, the mice were anesthetized, the chest cavity opened, and the mice perfused transcardially with 3 ml of 1000 units/ml of heparin in 0.1 M HEPES buffer (pH 7.3) followed immediately by 40 ml of 2.5% glutaraldehyde/0.5% paraformaldehyde in 0.1 M HEPES, pH

7.3, containing 0.1% picric acid. After the perfusion, the entire brain was then removed and placed in cold (4°C) fixative overnight. As mentioned above, the left half of the brain was used for probe placement verification, while the right half was kept for electron microscopic immuno-gold particle analysis. All tissue from each of the treatment groups was cut and processed on the same day so as to limit the variables that may occur by cutting and processing tissue on different days. Postembedding immuno-gold electron microscopy was performed according to the method of Phend et al. (1992), as modified by Meshul et al. (1994). The glutamate antibody, (nonaffinity purified, rabbit polyclonal; Sigma Chemical Co.), as previously characterized by Hepler et al. (1988), was diluted 1:200,000 in TBST 7.6. Aspartate (1 mM) was added to the glutamate antibody mixture 24 h prior to incubation with the thin-sectioned tissue to prevent any cross-reactivity with aspartate within the tissue. Photographs (10/animal) were taken randomly at a final magnification of 40,000× throughout the neurophil using a digital camera (AMT, Peabody, MA). The images were directly captured and stored on the computer by an individual blinded to the experimental groups. The glutamate immunolabeling technique was carried out for all of the treatment groups on the same day so as to limit the variables that may occur by carrying out this procedure on different days.

The number of gold particles per nerve terminal associated with an asymmetrical synaptic contact was counted, and the area of the nerve terminal determined using Image Pro Plus software (Media Cybernetics, Silver Springs, MD, Version 3.01). Glutamate-containing nerve terminals were typically photographed making a synaptic contact on a dendritic spine, indicating that they most likely originated from the motor cortex (Dube et al., 1988; Smith et al., 1994). The gold particles contacting the synaptic vesicles within the nerve terminal were counted and were considered part of the vesicular or neurotransmitter pool as previously established (Meshul et al., 1999). When nerve terminals contained mitochondria, gold particles associated with this organelle were not included as part of the final density measurement associated with that nerve terminal. The density of gold particles/$\mu m^2$ of nerve terminal was determined for each animal and the mean density for each group calculated (mean density ± SEM).

In addition to determining the density of nerve terminal glutamate immunolabeling, the same photomicrographs were used to calculate the mean percentage of asymmetrical synapses containing a discontinuous or perforated postsynaptic density (PSD) and the percentage of asymmetrical synapses forming multiple synaptic boutons (MSBs) (i.e., one terminal making contact with two or more dendritic spines) (Meshul et al., 2000). All asymmetrical synapses were counted if there was an accumulation of synaptic vesicles within the nerve terminal and there was a prominent postsynaptic density. A synapse was determined to contain a perforated PSD if there was a gap in the electron density of the PSD of more than 50 nm (the width of a synaptic vesicle) as previously defined by Greenough et al. (1978). Although Geinisman et al. (1991) has described three different shapes for perforated PSDs, the current study did not categorize the various perforated PSDs, since serial sectioning was not performed. The lack of serial sectioning most likely underestimates the percentage of all synapses with perforated PSDs. The mean percentage of all asymmetrical synapses with perforated PSDs (±SEM) or forming MSBs per 12 $\mu m^2$ was calculated.

The differences between the experimental groups were analyzed using the Student's *t*-test. The specificity of the immunolabeling for the glutamate antibody was previously established by incubating the antibody overnight with 3 mM glutamate (Meshul et al., 1994). This mixture was then applied to the sections as detailed above, with the final results showing a lack of tissue immunolabeling. The total number of synapses analyzed in the HR and LR groups was 162 and 170, respectively.

## RESULTS

### Behavioral testing

The mean number of line crossings for all animals over a 30-min-period was 767 ± 23. The mean number of line crossings for the HR group was 903 ± 18 and for the LR group was 653 ± 28 (Table I). Of the 15 mice tested, 8 had values above the mean and 7 had values below the mean.

### In vivo microdialysis

The mean basal extracellular concentration (pmole/μl) of striatal glutamate (±SEM) within the dialysate samples for the HR group was 1.17 ± 0.8, while the level for the LR group was 1.81 ± 0.09 (Table I). The difference between the two groups was statistically different. There was no difference in the basal extracellular level of striatal dopamine between the two groups (Table I). However, when the turnover ratio for dopamine was calculated [(DOPAC + HVA)/dopamine], there was a significant difference between the two groups (Table I). This difference of 73% between the HR and LR groups was statistically significant.

### Electron microscopy

Figure 1 shows nerve terminals making asymmetrical synaptic contacts with dendritic spines from both the HR and LR groups. There was about a 25% difference in the density of nerve terminal glutamate immunolabeling within the synaptic vesicle pool between

*TABLE I. Neurochemical and morphological measurements comparing the HR and LR groups*[a]

| | HR group | LR group | *P*-value |
|---|---|---|---|
| Mean number of line crossings/30 min | 903 ± 18[b] | 653 ± 28 | <0.0001 |
| Extracellular glutamate (pmole/μl) | 1.17 ± 0.08 | 1.81 ± 0.09 | <0.0001 |
| Extracellular dopamine (ng/ml) | 0.232 ± 0.06 | 0.257 ± 0.04 | <0.70 |
| (DOPAC + HVA)/dopamine | 87.7 ± 10.1 | 50.7 ± 7.3 | <0.047 |
| Density of nerve terminal glutamate immunolabeling (no. of gold particles/$\mu m^2$) | 89.5 ± 10.9 | 71.8 ± 9.2 | <0.25 |
| % of terminals making contact with a perforated PSD | 10.1 ± 2.1 | 4.7 ± 1.8 | <0.06 |
| % of terminals with MSB | 4.3 ± 2.9 | 1.7 ± 2.2 | <0.11 |

[a]Differences between the HR and LR groups were determined using the Student's *t*-test. Statistical significance was accepted at $P < 0.05$.
[b]Values are means ± SEM.

the two groups, with the HR group having a greater density compared with that of the LR group (89.5 ± 10.9 vs 71.8 ± 9.2, respectively) (Table I). However, this difference was not statistically different between the two groups. There was no difference in the size of the nerve terminals between the two groups (data not shown), suggesting that the small difference between the two groups in terms of nerve terminal glutamate immunolabeling was not due to changes in the area of the nerve terminals.

There was a more than 2-fold difference in the mean percentage of nerve terminals making a contact with a spine containing a perforated postsynaptic density (10.1 ± 2.1 vs 4.7 ± 1.8) and in the percentage of MSBs (4.3 ± 2.9 vs 1.7 ± 2.2) between the HR and LR groups, respectively (Table I). However, these differences were not statistically different.

## DISCUSSION

We report that in animals separated into two groups according to their activity in a novel environment, HR mice have a significantly lower level of extracellular glutamate within the dorsolateral striatum compared with that in the LR mice. At the ultrastructural level, we find a small but nonsignificant 25% difference in the density of glutamate immunogold labeling associated with the synaptic vesicle pool between the two groups. Although we had hypothesized that this lower level of extracellular striatal glutamate in the HR group could be due to a higher level of extracellular dopamine, we find no differences in striatal dopamine between the two groups. However, there is a higher degree of dopamine turnover, as determined by the ratio of dopamine metabolites to dopamine [(DOPAC + HVA)/dopamine] in the HR, compared to the LR group.

### In vivo microdialysis

On the basis of the report of an increase in the firing rate and bursting activity of dopamine neurons within the substantia nigra pars compacta neurons in the HR compared with that in the LR group (Marinelli and White, 2000), it was hypothesized that such an increase in dopamine neuronal activity in the HR group would lead to an increase in extracellular striatal dopamine, resulting in a decrease in the extracellular level of striatal glutamate. We report in the current study such a decrease in extracellular striatal glutamate in the HR, compared with that in the LR group, but no change in extracellular dopamine. The lack of change in dopamine levels between the HR and LR groups is consistent with previous reports, where no such change in basal dopamine between these groups was found within either the nucleus accumbens or striatum (Bradberry et al., 1991; Hooks et al., 1991, 1992; Piazza et al., 1991). When striatal tissue levels between the two groups were analyzed for both dopamine and its metabolite, DOPAC, there was an increase in the ratio of DOPAC/dopamine in the HR, compared with that in the LR group (Piazza et al., 1991). This finding is consistent with the higher ratio of (DOPAC + HVA)/dopamine found in the current study for the HR, compared with that of the LR group. Perhaps because of the higher turnover ratio for dopamine in the HR vs LR group, it has recently been reported that the LR group is more susceptible to the loss of dopamine following the injection of the neurotoxin, 6-hydroxydopamine, into the dorsolateral striatum (van Oosten and Cools, 2002).

Although it has been reported that dopamine agonist treatment results in a decrease in the release of glutamate from the dorsolateral striatum (Bamford et al., 2004), the finding in the current study of no change in extracellular striatal dopamine between the HR and LR groups suggests that the lower level of extracellular glutamate in the HR vs LR group is not due to a change in dopamine levels per se. However, an increase in dopamine turnover ratio in the HR, compared with that in the LR group, is consistent with an increase in firing rate and bursting activity of the substantia nigra pars compacta neurons (Marinelli and White, 2000). It is possible that such an increase in the dopamine turnover ratio may still be having an affect on the interaction between dopamine and its dopamine D2 receptor located on the glutamate nerve terminal. Considering that dopamine

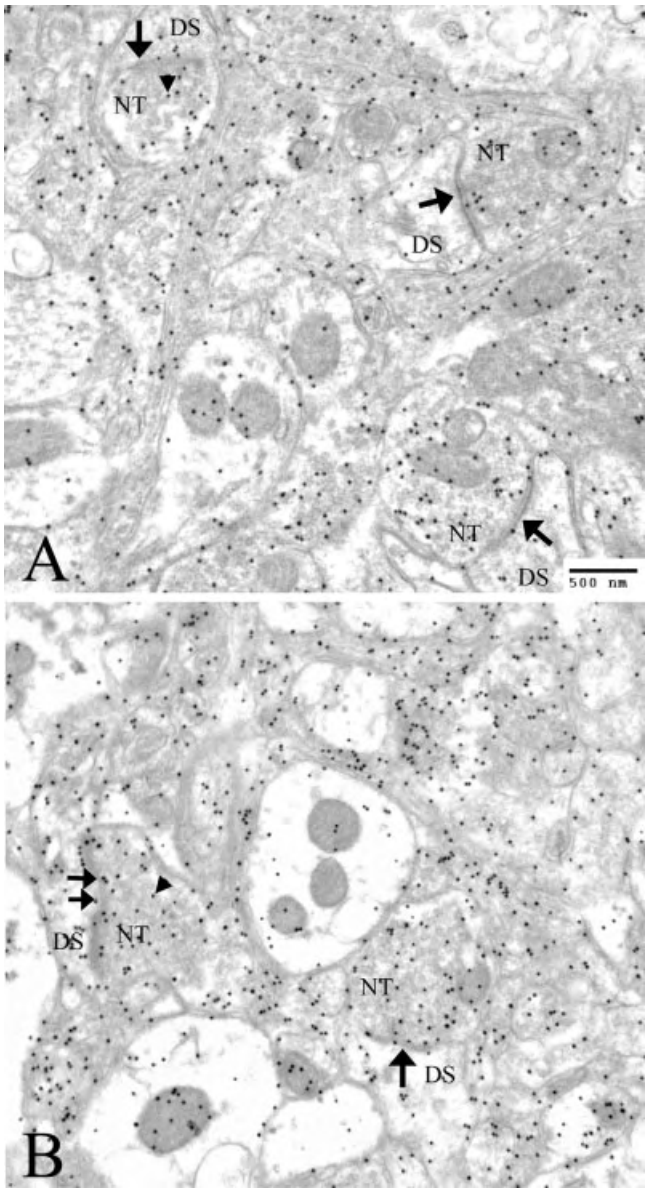


Fig. 1. Electron photomicrographs using the immuno-gold technique to localize an antibody against the neurotransmitter, glutamate, within the dorsolateral striatum. (**A**) LR group: within the nerve terminal (NT), there is an accumulation of small, round synaptic vesicles and 10-nm gold particles indicating the location of the antibody (arrowhead). The three nerve terminals are making synaptic contact (arrow) with an underlying dendritic spine (DS). (**B**) HR group: The two nerve terminals are making an asymmetrical synaptic contact with the underlying dendritic spine. The nerve terminal on the left side of the photomicrograph is making a contact with a spine that contains a perforated postsynaptic density (double arrows). The density of nerve terminal immuno-gold labeling is slightly greater than that seen in A above, but was not statistically significant (see Table I). Calibration Bar, 0.5 μm.

stimulation of these presynaptic receptors on glutamate nerve terminals results in a decrease in the release of glutamate, this would be consistent with the current findings of a decrease in the extracellular level of striatal glutamate in the HR, compared with that in the LR group.

It is also possible that there is a difference in the density of the glutamate transporter, GLT-1, between the HR and LR groups that might account for the differences in extracellular glutamate. GLT-1, as compared with the other glutamate transporters (GLAST, EAAC1, or EAAT4), is the major glutamate transporter that can affect extracellular glutamate levels (Maragakis and Rothstein, 2004; Suchak et al., 2003). We have recently reported a decrease in the relative density of GLT-1 immunolabeling within the striatum following acute MPTP administration, which was associated with an increase in the extracellular level of striatal glutamate (Meshul et al., 2003).

The HR and LR groups in the current study were not challenged with psychostimulants after the collection of baseline samples since we wanted to use the contralateral striatum from these same animals for further electron microscopic analysis. Future studies will focus on answering this question with regards to the effect of psychostimulants on extracellular striatal glutamate. In addition, the striatum from the probed side was not stimulated with potassium since we have previously reported that following the nearly complete loss of striatal dopamine (∼95%), due to a lesion of the nigrostriatal pathway, potassium depolarization of the lesioned vs nonlesioned striatum resulted in a similar increase in extracellular glutamate above baseline levels (Meshul et al., 1999). Therefore, in the current study in which there was no difference in extracellular striatal dopamine, it is very doubtful that potassium stimulation of the striatum would have resulted in any difference in extracellular glutamate between the HR and LR groups.

### Electron microscopy

Although we reported in the current study a difference in extracellular glutamate between the HR and LR groups, the 25% increase in the density of nerve terminal glutamate immunolabeling associated with the synaptic vesicle pool in the HR, compared with that in LR group, was not statistically different. We have reported that over 90% of the immuno-gold labeling within the nerve terminal is associated with the synaptic vesicle pool (Meshul et al., 1999). Therefore, the alterations in extracellular glutamate in the HR vs LR group may be associated with changes in either the cytoplasmic pool of glutamate or in the uptake of glutamate into glial cells. It has been reported that 60% of the basal extracellular level of glutamate is due to exchange with the glutamate/cystine antiporter, which has been shown to be calcium insensitive and most likely of cytoplasmic, but still of neuronal origin (Baker et al., 2002). We have reported that following a nearly complete loss of striatal dopamine (>95%) produced by a lesion of the nigrostriatal pathway with the neurotoxin, 6-hydroxydopamine

(6-OHDA), there is no change in the density of immuno-gold labeling within the cytoplasmic pool (Meshul et al., 1999). During the processing of the tissue, it is most likely that the vast majority of the cytoplasmic pool of glutamate is lost. It is not surprising that following a 6-OHDA lesion of the nigrostriatal pathway, there was no change in the density of the cytoplasmic pool of glutamate since it only accounted for less than 10% of the entire nerve terminal pool of glutamate. Therefore, it is possible that in the current study, the alterations in extracellular glutamate may be related to changes in the glutamate/cystine antiporter (Baker et al., 2002).

## CONCLUSIONS

It is well established that administration of psychostimulants results in changes in glutamate not only within the nucleus accumbens but also in the striatum (Kozell and Meshul, 2001; McKee and Meshul, 2005; Pierce et al., 1996; Wolf, 1998). Considering that animals displaying an increase in locomotor activity in a novel environment show an increase in self-administration of psychostimulants (Grimm and See, 1997; Piazza et al., 1989; Pierre and Vezina, 1997) and sensitization (Hooks et al., 1991, 1992; Pierre and Vezina, 1997), it is possible that the decrease in extracellular striatal glutamate in the HR group even before administration of any drugs of abuse may be an important factor that will contribute to this behavior. Indeed, it has been reported that alterations in glutamate function may be important for stereotypic behavior and drug-related habit formation (Hyman et al., 2001; Packard, 1999; Takahata and Moghaddam, 2003).

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# DIFFERENTIAL REGULATION OF THE GROWTH-ASSOCIATED PROTEINS GAP-43 AND SUPERIOR CERVICAL GANGLION 10 IN RESPONSE TO LESIONS OF THE CORTEX AND SUBSTANTIA NIGRA IN THE ADULT RAT

**E. J. HUGHES-DAVIS,[a,b]* J. P. COGEN,[b] M. W. JAKOWEC,[c] H. W. CHENG,[b] G. GRENNINGLOH,[d] C. K. MESHUL[e] AND T. H. McNEILL[a,b,c]**

[a]*Neuroscience Graduate Program, University of Southern California, Los Angeles, CA 90089, USA*

[b]*Department of Cell and Neurobiology, University of Southern California, Keck School of Medicine, Los Angeles, CA 90033, USA*

[c]*Department of Neurology, Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, USA*

[d]*Centre de Neurosciences Psychiatriques, Université de Lausanne, Site de Cery, CH-1008 Prilly, Switzerland*

[e]*Research Services, Neurocytology Lab, VA Medical Center and Dept. of Behavioral Neuroscience, Oregon Health and Science University, Portland, OR 97201, USA*

**Abstract—Investigation of the elements underlying synapse replacement after brain injury is essential for predicting the neural compensation that can be achieved after various types of damage. The growth-associated proteins superior cervical ganglion-10 and growth-associated protein-43 have previously been linked with structural changes in the corticostriatal system in response to unilateral deafferentation. To examine the regulation of this response, unilateral cortical aspiration lesion was carried out in combination with ipsilateral 6-hydroxydopamine lesion of the substantia nigra, and the time course of the contralateral cortical molecular response was followed. Unilateral cortical aspiration lesion in rats corresponds with an upregulation of superior cervical ganglion-10 mRNA at 3 and 10 days post-lesion, and protein, sustained from three to at least 27 days following lesion. With the addition of substantia nigra lesion, the response shifts to an upregulation of growth-associated protein-43 mRNA at 3 and 10 days post-lesion, and protein after 10 days. Nigral lesion alone does not alter contralateral expression of either gene. Likewise, motor function assessment using the rotorod test revealed no significant long-term deficits in animals that sustained only nigrostriatal damage, but cortical lesion was associated with a temporary deficit which was sustained when nigrostriatal input was also removed. Growth-associated protein-43 and superior cervical ganglion-10, two presynaptic genes that are postulated to play roles in lesion-induced sprouting, are differentially upregulated in corticostriatal neurons after cortical versus combined cortical/nigral lesions. The shift in contralateral gene response from superior cervical ganglion-10 to growth-associated protein-43 upregulation and associated behavioral deficit following combined cortical and nigral denervation suggest that nigrostriatal afferents regulate cortical lesion-induced gene expression and ultimate functional outcome. © 2005 Published by Elsevier Ltd on behalf of IBRO.**

**Key words: reactive synaptogenesis, cortical ablation, striatum.**

Unilateral destruction of the sensorimotor cortex has been a useful experimental model for investigating the cellular mechanisms that underlie synapse replacement after brain injury. Previous studies indicate that unilateral damage to the forelimb representation area of the rat cortex causes deficits in the use of the contralateral limb (Schallert et al., 2000), and is characterized anatomically by synapse loss followed by recovery on target neurons in both the ipsilateral striatum and contralateral cortex (Cheng et al., 1994; Jones et al., 1996; McNeill et al., 2003). In the striatum, sprouting of undamaged glutamatergic axon terminals originating from the contralateral homotopic cortex is governed by local neurotrophin and intracellular signals that are differentially responsive to various experimental and environmental factors (Cheng et al., 1998; Carmichael and Chesselet, 2002). In addition, markedly different patterns of lesion-induced gene expression and degrees of contralateral corticostriatal axon sprouting occur in the deafferented striatum depending on the mode of experimental brain damage (i.e. thermocoagulation vs. aspiration) (Szele et al., 1995; Napieralski et al., 1996; Snyder et al., 1998; Kartje et al., 1999; McNeill et al., 1999, 2003; Meshul et al., 2000; Uryu et al., 2001). However, while injury mode-dependent variation in the response to partial striatal deafferentation has been well documented, factors instrumental in determining the timing, pattern, and functionality of synapse replacement are not well established.

One factor that may be critical in modulating the compensatory response of corticostriatal neurons to brain injury is dopamine (DA). Previous anatomical studies indicate that DA neurons of the nigrostriatal and mesolimbic pathways form synapses on the necks of dendritic spines of striatal and cortical neurons that also receive glutamatergic input (Bjorklund and Hokfelt, 1984; Bouyer et al., 1984; Groves et al., 1994; Smith et al., 1994). Evidence that DA can modulate the activity of target neurons is well documented in the literature (Haracz et al., 1998; Centonze et al., 1999; Cepeda et al., 2001), suggesting that

*Correspondence to: E. J. Hughes-Davis, Department of Cell and Neurobiology, University of Southern California, Keck School of Medicine, BMT 401, 1333 San Pablo Street, Los Angeles, CA 90033, USA. Tel: +1-323-442-2126; fax: +1-323-442-2127.
E-mail address: elizabjd@usc.edu (E. J. Hughes-Davis).

*Abbreviations:* BDNF, brain-derived neurotrophic factor; CX, cortex lesion; DA, dopamine; GAP-43, growth-associated protein-43; MSB, multiple synaptic bouton; SCG10, superior cervical ganglion-10; SN, substantia nigra lesion; 6-OHDA, 6-hydroxydopamine.

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optimal modulation of corticostriatal strength by DA is critical for maintaining various cognitive and motor functions in adults. In addition, dysfunction of dopaminergic signaling is central to the etiopathology of various neurological disorders such as schizophrenia (Goldman-Rakic et al., 2004; Harrison and Weinberger, 2005), Parkinson's disease (Jellinger, 2001), and drug addiction (Koob and Nestler, 1997). Furthermore, DA, working through specific subtypes of DA receptors, has been shown to play an important role in the regulation of neuronal excitability and synaptic plasticity of striatal neurons that form both the direct and indirect output pathways, and can also exert a widespread effect on cortical function (Cepeda and Levine, 1998; Steiner and Kitai, 2000; Centonze et al., 2001, 2003; Jay, 2003). Given the proposed role of DA in maintaining cortical and striatal activity, we wanted to determine whether DA signaling may also play an important role in modulation of lesion-induced gene expression thought to underlie neurite outgrowth and synaptogenesis in the striatum after brain injury.

To test the hypothesis that dopaminergic inputs regulate lesion-induced gene expression, we carried out a series of analyses on rats that underwent three different striatal deafferentation lesions. These included: a unilateral aspiration lesion of the primary motor cortex (CX), a unilateral lesion of dopaminergic neurons originating in the substantia nigra (SN) using the neurotoxin 6-hydroxydopamine (6-OHDA), or a combination lesion of both the cortex and substantia nigra (CX/SN). We hypothesized that the loss of nigrostriatal afferents in combination with unilateral cortex ablation would lead to a spontaneous response marked by an aberrant pattern of growth associated gene expression, which may underlie the unusual pattern of synapse replacement observed in our previous anatomical study using this combined lesion (Meshul et al., 2000). Furthermore, as a consequence of these atypical molecular and anatomical changes, we hypothesized that motor function recovery would be dampened in animals with CX/SN lesions.

For this study, the response of corticostriatal afferents contralateral to the lesion, which make up the primary population of reactive fibers (Napieralski et al., 1996; Cheng et al., 1998; McNeill et al., 1999), was assessed with regard to expression of two growth-associated protein genes previously reported to be linked with reactive synaptogenesis in the deafferented striatum: growth-associated protein-43 (GAP-43) and superior cervical ganglion-10 (SCG10). SCG10 is a neuron-specific, membrane-associated phosphoprotein that is highly concentrated in the growth cones of developing neurites (Stein et al., 1988; Di Paolo et al., 1997). It is a member of the stathmin gene family and is proposed to play a key role in linking growth cues to the regulation of microtubule dynamics during neurite extension (Marklund et al., 1996; Riederer et al., 1997). SCG10 mRNA is increased in a time dependent manner in contralateral cortex neurons following a unilateral aspiration lesion of the cortex (McNeill et al., 1999). Similarly, GAP-43, a fast axonally transported phosphoprotein, is induced during axonal growth in both early development and regeneration. It has been proposed to play a key role in signal transduction pathways that mediate pathfinding signals from the growing neurite (Skene and Virag, 1989; Strittmatter et al., 1995) and is often used as a marker for the sprouting response to injury (Stroemer et al., 1993; Li et al., 1998; King et al., 2001). mRNA levels for both genes were assessed by *in situ* hybridization across the three lesion types at 3, 10, and 27 days post-lesion. In addition, Western blot analysis was used to examine changes in the levels of GAP-43 and SCG10 protein in the contralateral cortex. Finally, molecular changes were correlated with the recovery of coordinated sensorimotor function, as measured by the rotorod test.

## EXPERIMENTAL PROCEDURES

Young adult male Fischer 344 rats (225–250 g, Harlan Sprague–Dawley, Indianapolis, IN, USA), were housed in a temperature-controlled 12-h light/dark cycle standard facility with free access to dry food and water. Experimental groups consisted of rats killed at 3, 10, or 27 days after either unilateral cortex ablation, a unilateral 6-OHDA lesion of the substantia nigra or a combination of both lesion types ($n$=5 rats per post-lesion time point). The rotorod test was used to assess coordinated motor function among different experimental groups and controls. Rats were tested at the beginning of the study and before being killed at 3, 10, or 27 days post-lesion. While in the vivaria, animal care followed the guidelines set forth by the NIH Guide for the Care and Use of Laboratory Animals, and the Institutional Animal Care and Use Committee at the University of Southern California approved all experimental protocols. All efforts were made to minimize animal suffering and the number of animals used.

### Lesions

Unilateral CXs were made as described previously (Cheng et al., 1994; McNeill et al., 1999, 2003). Briefly, while under ketamine (80 mg/kg)/xylazine (5 mg/kg, i.p.) anesthesia, cortical ablation was performed by removing part of the skull over the right side of the brain, incising the dura, and aspirating the primary motor representation area of the sensorimotor cortex with a fine-tipped glass pipette. Care was taken not to injure the underlying corpus callosum (Fig. 1). In addition, a group of animals underwent sham procedures in which part of the skull and dura were removed, but the underlying cortex was not disturbed.

Unilateral lesions of the right nigrostriatal dopaminergic pathway were made using the neurotoxin 6-OHDA, as described pre-

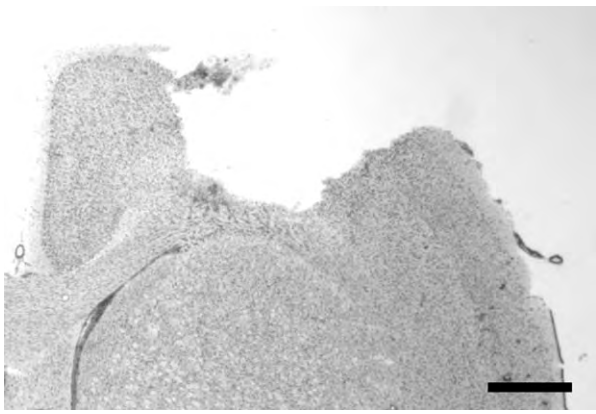

**Fig. 1.** Photomicrograph of Cresyl Violet-stained representative cortical aspiration lesion. Scale bar=1 mm.

viously (Schauwecker et al., 1998). Briefly, unilateral injections of 6-OHDA (1 mg/ml in 0.1% ascorbic acid dissolved in sterile saline, Sigma, St. Louis, MO, USA) were made in two locations: the rostral SN pars compacta (from Bregma, AP: −4.8 mm; ML: +1.7 mm; DV: −8.5 mm from top of skull) and the medial forebrain bundle (AP: −4.3 mm; ML: 1.2; DV: −8.5 mm). 6-OHDA was infused at a rate of 1 μl/min for 3 min at each site. For the combined CX/SN group, both surgeries were performed at the same time. Sham-operated controls received vehicle injections without the toxin at the same anatomical locations as noted above. Rats undergoing unilateral cortex ablation or the SN lesion recovered without postsurgical complications.

At 3, 10, and 27 days post-lesion, rats were killed by decapitation under anesthesia and a tissue block of the homotopic cortex contralateral to the lesion was dissected and rapidly frozen in isopentane on dry ice and stored at −80 °C before being processed for Western blot analysis. A separate set of rats was used for the *in situ* hybridization study: for these animals, the brain was rapidly removed and frozen whole in isopentane and then stored at −80 °C for later analysis.

To verify the effectiveness of the 6-OHDA lesion, the midbrains from SN and CX/SN groups were blocked fresh, immersion fixed in 4% paraformaldehyde and processed for tyrosine hydroxylase immunocytochemistry and Cresyl Violet staining (Fig. 2, Schauwecker et al., 1998; Jakowec et al., 2004). Sections were cut on a sliding microtome at a thickness of 30 μm and a systematic random sample of every fifth section was collected and stained for tyrosine hydroxylase immunoreactivity (Chemicon, Temecula, CA, USA) and counterstained for Nissl substance. The number of nigrostriatal dopaminergic neurons in the substantia nigra pars compacta was determined by unbiased stereology with the computer-imaging program Bioquant Nova Prime (Bioquant Imaging, Nashville, TN, USA) and an Olympus BX-50 microscope equipped with a motorized stage and CCD camera. Each ventral mesencephalon section was viewed at low magnification (×10 objective) and the substantia nigra pars compacta outlined and delineated, based on tyrosine hydroxylase immunoreactivity, from the ventral tegmental-immunoreactive neurons using the third nerve and cerebral peduncle as landmarks. Neurons were viewed at high magnification (×80 objective) and counted if they displayed tyrosine hydroxylase immunoreactivity and had a clearly defined nucleus and cytoplasm. The total number of substantia nigra pars compacta dopaminergic neurons was determined based on the method of Gundersen and Jensen (1987), and only rats with 95% DA cell loss or greater were used in these studies.

## Riboprobe preparation

Probes were prepared as described by Schauwecker et al. (1995). All cRNA probes used in the present study were transcribed in the presence of [$^{35}$S]-UTP (1300 Ci/mmol; Dupont, Wilmington, DE, USA). Radiolabeled GAP-43 cRNAs were transcribed from a plasmid containing an 1121 base pair rat GAP-43 insert (provided by Dr. M. Fishman, Harvard University, Cambridge, MA, USA). SCG10 riboprobe was transcribed from a vector containing a 600 base pair rat SCG10 insert (provided by Dr. G. Grenningloh, University of Lausanne, Prilly, Switzerland).

## *In situ* hybridization

*In situ* hybridization was performed using the method outlined previously (McNeill et al., 1999). Sixteen-micrometer thick sections from the frozen brains of rats ($n$=5 per time point) decapitated at 3, 10, and 27 days post-lesion were thaw-mounted on poly-L-lysine-coated slides. Frozen sections were stained with Cresyl Violet to document the size and location of the CX, or, alternatively, fixed in fresh 4% paraformaldehyde, treated with 0.1 M triethanolamine, and immersed in triethanolamine with acetic anhydride. Sections were then dehydrated, air dried, and hybridized overnight at 50 °C with [$^{35}$S]-labeled antisense cRNA. For emulsion autoradiography, slides were dipped in NTB-2 emulsion (Eastman Kodak, Rochester, NY, USA; diluted 1:1 in 600 mM ammonium acetate) and exposed at 4 °C for 3–5 weeks. The slides were developed in D-19 (Eastman Kodak), fixed, and counterstained with Cresyl Violet.

We examined changes in mRNA levels at three post-lesion time points, and compared these findings with data from unlesioned sham-operated rats. The cortical lesions removed primary motor areas of sensorimotor cortex, including forelimb cortical areas (McGeorge and Faull, 1989; Paxinos and Watson, 1997; McNeill et al., 1999). We confined our analysis of grain density to neurons in layer V because previous work has shown that pyramidal neurons in this region predominate the contralateral corticostriatal projection (Akintunde and Buxton, 1992; McNeill et al., 1999; Reiner et al., 2003).

Quantification of grain density over individual cell bodies was assessed using computer-enhanced video densitometry and image analysis software (Bioquant). Cells were randomly selected and the number of grains counted with a 100× oil-immersion objective lens. In order to randomly select cells, a grid pattern was placed over the field of interest in the image analysis program, and cells that were located at an intersection of the grid were selected for counting if they also had grain accumulation over five times the background value. For each animal, 15 cells from five randomly

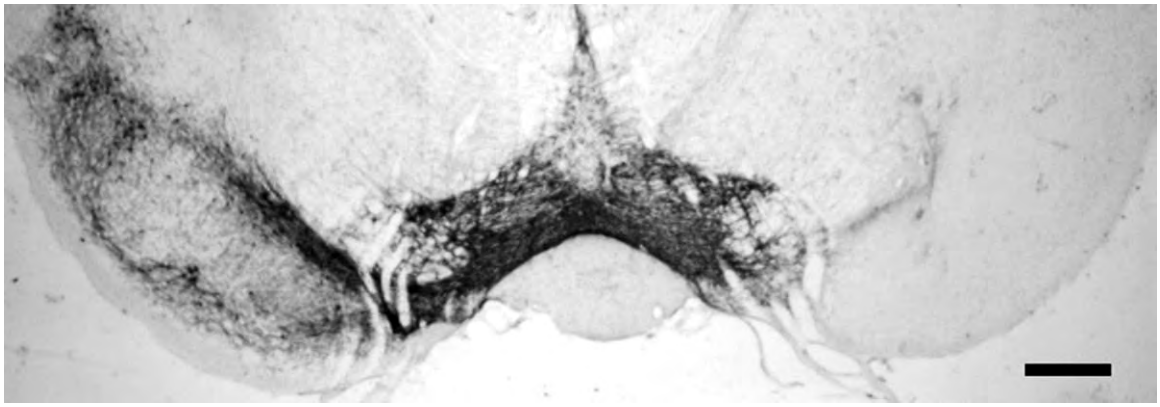

**Fig. 2.** Photomicrograph of representative tyrosine-hydroxylase immunostained coronal slice, shows extent of unilateral 6-OHDA lesion of the substantia nigra. Scale bar=1 mm.

selected tissue sections between the anatomical levels IA 10.00–IA 9.48 (Paxinos and Watson, 1997) were analyzed.

### Western blot

Rats ($n$=5 per time point) were decapitated, the brains quickly removed, and contralateral cortices rapidly dissected and frozen. Protein was extracted from the tissue using lysis buffer (137 mM NaCl; 20 mM Tris, pH 8.0; 1% Triton X-100; 10% glycerol; 1 mM phenylmethylsulfonyl fluoride, 500 μM orthovanadate). Protein concentrations were measured using the Bio-Rad protein assay kit (Hercules, CA, USA). Protein samples (50–75 μg) were mixed with sodium dodecyl sulfate containing sample buffer, heated for 3 min at 96 °C and electrophoresed on a 10% sodium dodecyl sulfate–polyacrylamide gel (Laemmli, 1970). Separated proteins were transferred to a nitrocellulose membrane, which was blocked with 5% nonfat dry milk in Tris-buffered saline (10 mM Tris–HCl, pH 7.4; 0.9% saline) for 3 h at 37 °C. Blots were then incubated in GAP-43 (Sigma) or SCG10 (generously provided by Dr. G. Grenningloh) primary antisera for 16 h at 4 °C in Tris-buffered saline containing 0.2% Tween-20 and 0.2% bovine serum albumin, followed by incubation in secondary antibody–horseradish peroxidase conjugate in Tris-buffered saline containing 0.2% Tween-20. Visualization of the site of antigen-antibody binding was performed with Kodak X-Omat film and ECL Western Blotting Detection agents (Amersham Life Science, Buckinghamshire, UK) according to the supplied procedure. Bands were quantified using computer-assisted video densitometry (Image Measure, Microscience, Federal Way, WA, USA).

### Motor function testing

Coordinated motor function was tested by measuring each animal's ability to balance on a rotating rod (Omni-roto, Omnitech Electronics, Columbus, OH, USA). This test was selected because the corticostriatal pathway plays an important role in the coordination of sequenced motor activity (Alexander and Crutcher, 1990; Middleton and Strick, 2000) and unilateral damage to the primary motor representation area of the sensorimotor cortex causes subtle deficits in the use of the contralateral forelimb, making it difficult to maintain balance on the rotating rod. The rotorod test has been used with rats to identify age-related declines in motor activity (Wallace et al., 1980) and to assess recovery of function after cortical injury or striatal treatments (Emerich et al., 1993, 1996; Meshul et al., 2000). Rats were trained for 1–2 days prior to surgery. During four trials, separated by 30 minute rest periods, rats were placed on the rod while it was stationary and then tested at fixed speeds of 3, 6, 9, and 12 rotations/min. After training, animals were able to balance on the rotorod for at least 120 s (cutoff). Immediately prior to kill, each rat was given four trials; the longest run time was used for analysis.

### Statistical analysis

Results of *in situ* hybridization, Western blot, and behavioral tests were assessed statistically using one-way ANOVA, and intergroup differences analyzed by Newman-Keuls post hoc test, with $P<0.05$.

## RESULTS

### SCG10 and GAP-43 mRNA distribution

The distributions of mRNAs for SCG10 and GAP-43 in sham-operated animals were similar to those in earlier reports (Himi et al., 1994; McNeill et al., 1999; Ozon et al., IIIIII). No differences were found between CX-sham rats and nigral-sham rats, so their values were pooled. Cortical neurons labeled for SCG10 mRNA were distributed in all cortical layers except layer I. The heaviest label was found in

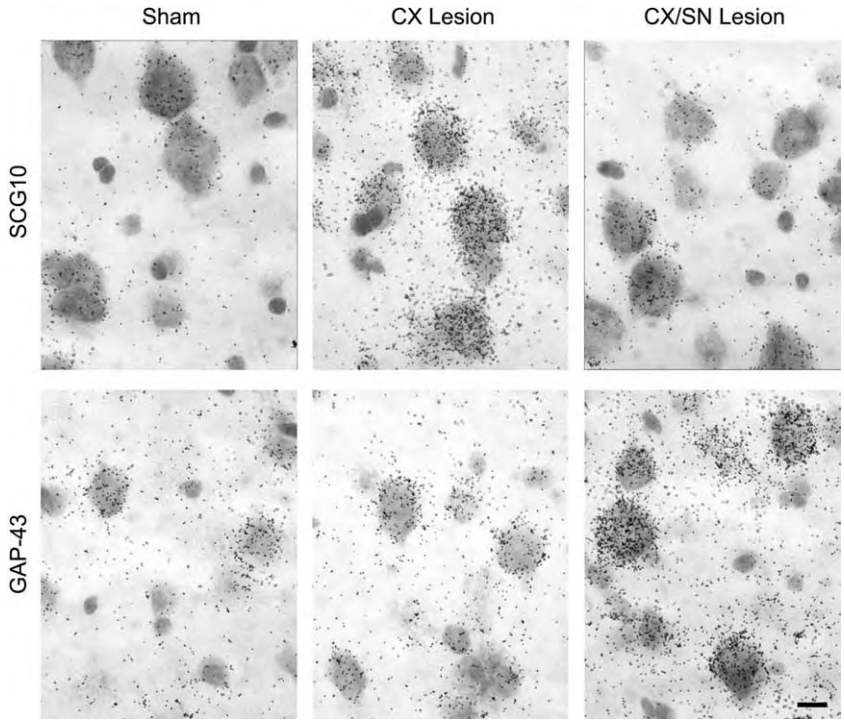


**Fig. 3.** Representative photomicrographs showing the changes in hybridized grain density in layer V contralateral cortex for the growth associated proteins at the 3 day time point after unilateral CX and CX/SN lesions. Top panel: increase in SCG10 mRNA of a CX-lesioned rat relative to sham. Bottom panel: increase in GAP-43 mRNA expression in a CX/SN-lesioned rat. Images have had contrast and brightness uniformly adjusted. Scale bar=10 μm.

the large pyramidal cells of layer V, and in medium sized neurons in layers III, V, and VI. GAP-43 label was confined mainly to infragranular layers of the cortex with the densest labeled cells located in layer V. Levels of both mRNAs were elevated in motor cortex in contrast to more lateral areas of the somatosensory cortex. Semi-adjacent tissue sections hybridized with the sense probe showed no significant label.

### Lesion-induced response determined by *in situ* hybridization

As in previous work, we found a significant increase in the level of SCG10 mRNA in layer V pyramidal neurons of the contralateral homotopic cortex after the unilateral CX lesion, but no change in GAP-43 mRNA (McNeill et al., 1999). Specifically, SCG10, but not GAP-43, mRNA was significantly increased at three days post-lesion and decreased to sham-operated values at 27 days post-lesion. In contrast, upregulation of GAP-43, but not SCG10, mRNA was seen in the contralateral cortex after the combined CX/SN lesion (Fig. 3). GAP-43 grain density was increased maximally in the CX/SN group at 3 days post-lesion (Fig. 3; $P<0.05$), remained significantly elevated after 10 days, and fell back to control levels by 27 days (Fig. 4). Changes in mRNA for GAP-43 were specific to homotypic areas of cortex on the contralateral side, but there were no significant alterations of SCG10 mRNA in the contralateral cortex after the CX/SN lesion (Fig. 4). Furthermore, the levels of SCG10 and GAP-43 mRNA in pyramidal neurons of the contralateral cortex remained unchanged at all time points after the SN lesion.

### Lesion-induced response determined by Western blot

SCG10 and GAP-43 protein changes after the unilateral CX have been described previously (McNeill et al., 1999), and, as with mRNA, we found a time dependent increase in the level of SCG10 but not GAP-43 protein in the contralateral cortex. Specifically, the level of SCG10 protein was increased at 3 days post-lesion and remained elevated above sham-operated values at 27 days post-lesion. However, after the combined CX/SN lesion, the pattern was reversed: GAP-43 protein levels increased significantly by 10 days post-lesion, before returning to control levels at 27 days post-lesion (Fig. 5), but SCG10 protein levels showed no significant change after the CX/SN lesion. In addition, as with the mRNA data, we found no change in the level of SCG10 or GAP-43 protein in the contralateral cortex after SN lesion alone.

### Motor function analysis

We found that the amount of time rats with unilateral CXs maintained their balance and remained on the rotorod was significantly reduced at 3 and 10 days post-lesion, but recovered to 80% of intact values after 27 days. Rats with the combined CX/SN lesion also showed a significant reduction in time on the rotorod at 3 and 10 days post-lesion, but, in contrast, showed only a modest recovery of motor function compared with sham-operated rats at the 27 day time point. In addition, the recovery of motor function in the CX/SN group was significantly below that observed in rats of the CX group (Fig. 6). In comparison, similar to what has been described by other investigators (Whishaw et al., 2003), rats with the SN lesion alone showed only a mild reduction in motor performance at 3 days post-lesion that was not statistically different from unlesioned rats. By 10 days post-lesion, coordinated motor function for rats with the SN lesion, as measured by the rotorod test, was the same as for sham-operated rats.

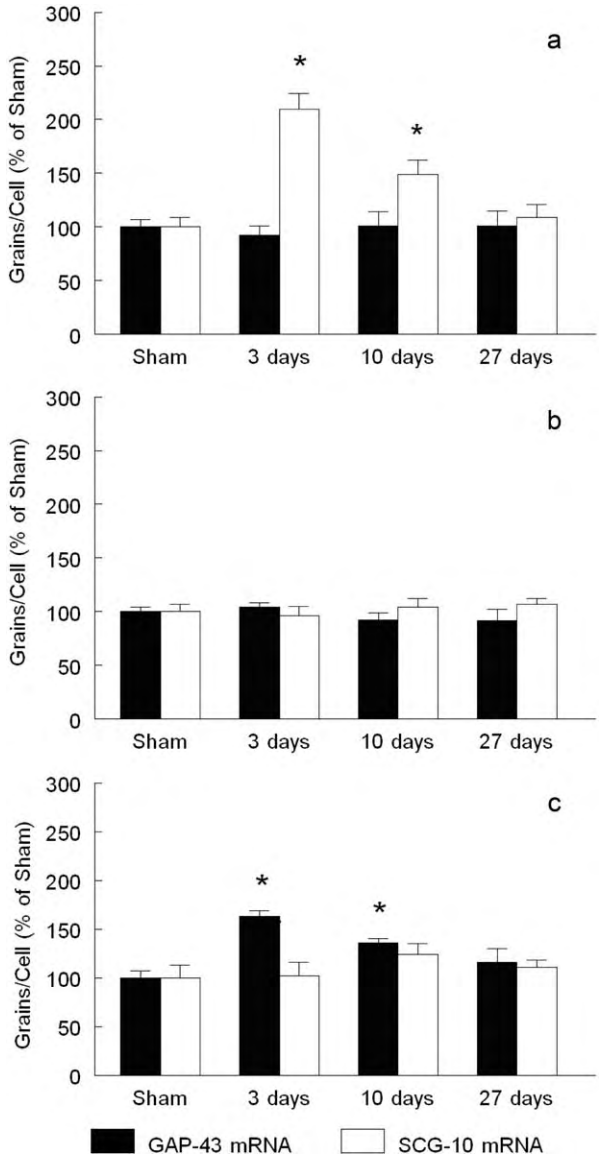


**Fig. 4.** Quantification of GAP-43 and SCG10 mRNA by *in situ* hybridization in layer V neurons of the contralateral cortex after CX (a), SN (b), and CX/SN (c) lesions. Means per group are expressed as a percentage of the number of grains per neuronal cell body found in sham animals±S.E.M.; * $P<0.05$ compared with sham value.

## DISCUSSION

These studies add to our growing understanding of the heterogeneity of neuronal responses that occur after brain

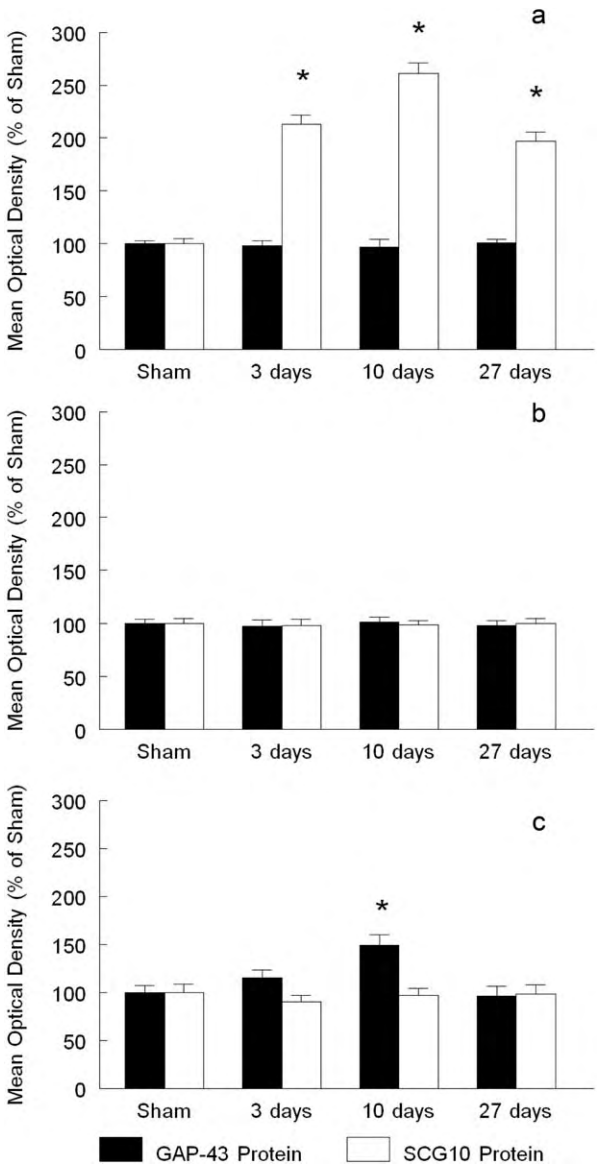


**Fig. 5.** Expression of GAP-43 and SCG10 protein determined by Western blot in contralateral cortex after CX (a), SN (b), and CX/SN (c) lesions. Means per group are expressed as a percentage of the GAP-43 or SCG10 mean optical density found in sham animals±S.E.M.; * $P<0.05$ compared with sham value.

injury. Specifically, our results provide evidence that the two growth-associated proteins SCG10 and GAP-43 are differentially induced in contralateral cortex neurons as a result of different deafferentation conditions and that neurons of the nigrostriatal pathway most likely play a role in modulating this response. This finding is consistent with previous studies that have reported that different post-lesion milieus, caused by different types of damage, induce markedly different patterns of lesion-induced gene expression (Szele et al., 1995; McNeill et al., 1999) and supports the notion that brain damage does not induce a generic response mechanism or outcome. Likewise, our findings suggest that the differential regulation of specific sets of growth-associated genes following brain injury may play a central role in defining the degree and final pattern of reinnervation achieved in the reconstructed striatum (Mason et al., 2002, 2003). Previous studies from our laboratory found that after cortical lesion alone, the reconstructed striatum is characterized by an upregulation of SCG10 and a six-fold increase in the number of terminals that contact more than one dendritic spine (multiple synaptic boutons, MSBs), a feature not normally found in the intact brain (Meshul et al., 2000; McNeill et al., 2003). In comparison, additional loss of nigrostriatal input is associated with an upregulation of GAP-43, and approximately double the asymmetrical terminals forming MSBs (14-fold increase over control). These findings are of interest because previous studies suggest that MSBs play an important role in experience induced synaptic plasticity (Sorra and Harris, 1993; Harris, 1995; Woolley et al., 1996; Jones, 1999; Toni et al., 1999; Nikonenko et al., 2002), and may lead to activity-dependent changes in the reorganized striatum that can facilitate functional recovery. Identifying the factors that are specifically associated with the loss of nigrostriatal neurons is an important step in providing a better understanding of the cellular mechanisms that modulate neuroplasticity after various types of brain injury.

The loss of input, either physical or chemical, from nigrostriatal neurons in combination with cortical lesion leads to a pattern of reactive gene expression that is temporally correlated with a different pattern of synapse formation than that following cortical lesion alone, and also appears to limit the recovery of motor function. It is possible that the response to the combined lesion, characterized by GAP-43 induction and the large increase in MSBs, represents an exaggerated effort by the nervous system to compensate for more extensive physical damage inherent in loss of both cortical and nigral fibers. However, the relative distribution of these afferent systems in the striatum is disproportionate, with the cortex contributing about half of all asymmetrical axo-spinal striatal synapses and the SN less than 10% of that number (Wilson et al., 1983). Furthermore, nigral terminations occur only on spines that also have asymmetrical contracts, so an expansion of the cortically-denervated target area is likely to be minimal with the addition of unilateral SN lesion (Wilson et al., 1983). Alternatively, factors associated with nigral input, such as DA, may contribute to mechanistic regulation after cortical lesion, and play an important role in regulating gene expression and synapse formation after damage to the sensorimotor cortex. The convergence of dopaminergic and glutamatergic pathways within the striatum is extensively documented. Not only do striatal medium spiny neurons receive both types of afferents, excitatory terminals often ramify on the same dendritic spines as dopaminergic terminals (Bouyer et al., 1984; Groves et al., 1994; Smith et al., 1994). Because convergent dopaminergic and glutamatergic systems in the intact striatum influence each other in several ways, the tri-part pattern of connectivity in the striatum may influence the circuit response after corticostriatal lesion (Cepeda and Levine, 1998; Centonze et al., 2001). Indeed, DA is a likely reinforcing component in corticostriatal synaptic plasticity (Reynolds et al., 2001),

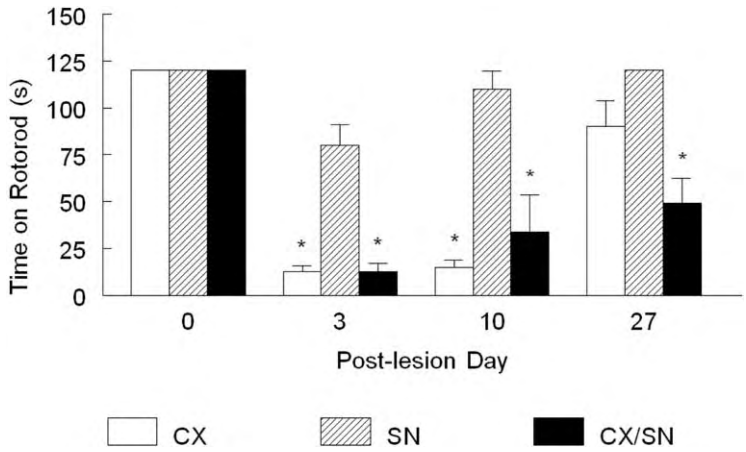


**Fig. 6.** Rotorod test performance during post-lesion time course after CX, SN, and CX/SN lesions. Mean time spent on the rotorod±S.E.M.; * $P<0.05$ compared with sham value.

and DA manipulations influence corticostriatal synaptic configurations (Meshul and Tan, 1994; Meshul et al., 1999; Meshul and Allen, 2000; Avila-Costa et al., 2005). Striatal dopaminergic and corticostriatal glutamatergic systems coordinate to maintain an appropriate balance through multiple points of interaction; thus, it is plausible that manipulating one side of this arrangement will elicit an adaptation from the other. These studies highlight the importance of investigating the functional consequences of manipulating DA activity after brain injury as part of an overall treatment strategy to facilitate the recovery of motor function.

Our finding that recovery of motor function after brain injury is dependent on the post-lesion signaling environment suggests that functional recovery after damage to the sensorimotor cortex is related to the reactive molecular and anatomical phenomena described in these studies. Specifically, previous studies from our laboratory (Meshul et al., 2000) and those of others (Sutton et al., 1989; Goldstein, 1993) have reported that the synaptic circuitry of the reconstructed striatum, although different from that of the intact striatum, can functionally compensate for the loss of ipsilateral cortical input. The sustained behavioral deficit reported here following CX/SN lesion suggests that nigrostriatal activity normally contributes to the ability to compensate after cortical lesion. The lack of general body coordination observed on the rotorod may be caused by asymmetrical postural supports or sensation, for example, and calls for more selective behavioral interventions and assessments (Schallert et al., 2000; Whishaw et al., 2003). We believe the difference in motor function with regard to ultrastructural changes following cortex aspiration alone and CX/SN lesions suggests there may be an optimal range at which MSBs impart functional utility: an overabundance of MSBs may represent unregulated miswiring of motor circuits, whereas in moderation, MSBs may offer a basic framework upon which to build feedback-dependent, functionally relevant networks. As in development, more is not necessarily better, and an appropriate balance of synaptic contacts is required to establish synaptic circuitry that can lead to motor recovery.

Although DA is the most likely candidate to modulate the compensatory response of corticostriatal neurons to brain injury, other factors may also be responsible for determining the timing and pattern of synapse replacement. For instance, the nigrostriatal pathway also supplies the striatum with brain-derived neurotrophic factor (BDNF, Altar et al., 1997) which has been shown to influence survival, sprouting, and synaptogenesis in different neural systems (Hammond et al., 1999; Mamounas et al., 2000; Alsina et al., 2001). Previous studies have reported that BDNF plays a role in axonal and dendritic remodeling in the developing CNS (Cohen-Cory and Fraser, 1995; Cabelli et al., 1997; Lom and Cohen-Cory, 1999; Lein and Shatz, 2000) and the sustained expression of BDNF in many regions of the adult brain suggests that it also plays an essential role in the maintenance of synaptic circuitry throughout the lifetime of the animal. In mature animals, studies have shown that BDNF has profound effects on neurotransmission, activity-dependent synaptic remodeling, and neurogenesis. It also provides a means of neurotrophic support to striatal neurons after brain injury (Altar and DiStefano, 1998). Furthermore, stimulation of signaling pathways downstream of BDNF activity are known to affect cytoskeletal elements including microtubules and actin, which may underlie its growth-inducing function, as well as enhance neuron survival and plasticity of deafferented or injured neurons after brain damage (reviewed in Atwal et al., 2000). Rather than exclusive effects of either DA or BDNF on the reinnervation response, continuous interplay among afferent signaling systems, including DA, BDNF, and glutamate, is likely to refine the growth response throughout the period of reinnervation. The results presented suggest that control of corticostriatal presynaptic growth-associated genes and the resulting functional recovery are modulated at least in part through a nigrostriatal-dependent mechanism. Further studies are needed to resolve whether these responses are simply damage-dependent or represent valid mechanisms, perhaps involving DA or BDNF, by which the brain can produce functionally relevant reinnervation.

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# Behavioral Motor Recovery in the 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine-Lesioned Squirrel Monkey (*Saimiri sciureus*): Changes in Striatal Dopamine and Expression of Tyrosine Hydroxylase and Dopamine Transporter Proteins

**Giselle M. Petzinger,[1,2]* Beth Fisher,[2] Elizabeth Hogg,[1] Avery Abernathy,[1] Pablo Arevalo,[1] Kerry Nixon,[1] and Michael W. Jakowec[1,2]**

[1]George and MaryLou Boone Parkinson's Disease and Movement Disorders Research Center, Department of Neurology, University of Southern California, Los Angeles, California
[2]Department of Biokinesiology and Physical Therapy, University of Southern California, Los Angeles, California

The neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) provides an excellent opportunity to study repair and response to injury in the basal ganglia. Administration to mammals leads to the destruction of nigrostriatal dopaminergic neurons and depletion of striatal dopamine. In the squirrel monkey (*Saimiri sciureus*), MPTP-lesioning results in parkinsonian motor symptoms including bradykinesia, postural instability, and rigidity. Over time animals display motor behavioral recovery. To better understand this mechanism we employed a lesioning regimen of two or six subcutaneous injections of MPTP (2.0 mg/kg, free-base) to generate mild or moderate parkinsonism. Brain tissue was harvested at 6 weeks or 9 months after the last injection and analyzed for dopamine and its metabolites by high performance liquid chromatography (HPLC), and by immunohistochemical staining and Western immunoblotting for the expression of tyrosine hydroxylase (TH), dopamine transporter (DAT), and dopamine- and cAMP-responsive protein phosphatase of 32 kDa (DARPP-32), an effector molecule enriched in striatal medium spiny neurons. Several months after MPTP-lesioning, when squirrel monkeys displayed full motor behavioral recovery, striatal dopamine levels remained low with a greater return in the ventral striatum. This finding is consistent with other reports using neurotoxicant-lesioning models of the basal ganglia in rodents and other species of nonhuman primates. Elevated dopamine turnover ratio and decreased DAT expression appeared in early behavioral recovery at the 6-week time point in both mild- and moderate-parkinsonian monkeys. Tyrosine hydroxylase and DAT expression was increased in late stage recovery even within dopamine-depleted regions and supports sprouting. Altered DARPP-32 expression suggests a role of medium spiny neurons in recovery. 

**Key words:** basal ganglia; Parkinson's disease; nonhuman primate; neuroplasticity

The neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) provides an excellent opportunity to study repair and the response to injury in the basal ganglia. MPTP is a meperidine derivative that can be administered systemically, and the pre-toxin form crosses the blood–brain barrier and is converted to the toxic form 1-phenyl-4-phenylpiperidium ($MPP^+$) by astrocytic monoamine oxidase B (Chiba et al., 1985). $MPP^+$ acts as a "false-substrate" for the dopamine transporter (DAT) and accumulates in dopaminergic neurons where it targets mitochondrial complex I leading to energy depletion and the formation of reactive oxygen

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*Correspondence to: Giselle M. Petzinger, MD, Department of Neurology, Keck School of Medicine, 1333 San Pablo St., MCH-148, Los Angeles, CA, 90033. E-mail: gpetzinger@surgery.usc.edu

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species (Dauer and Przedborski, 2003). This results in the selective destruction of nigrostriatal dopaminergic neurons and the depletion of the striatal neurotransmitter dopamine similar to that seen in Parkinson's disease (PD) (Jakowec and Petzinger, 2004). Dopamine depletion results in behavioral motor deficits in mice and nonhuman primates. Motor behavioral changes in mice tend to be subtle and require specific behavioral testing including treadmill, paw-reach, and rotorod balancing to become evident (Sedelis et al., 2000, 2001; Tillerson et al., 2001; Fisher et al., 2004). In nonhuman primates, motor behavioral deficits resembling those seen in humans with PD or drug addicts who self-administered MPTP are evident and include akinesia, bradykinesia, postural instability, freezing, and in some species a resting tremor (Burns et al., 1983; Langston et al., 1983, 1984).

MPTP has been administered to a variety of different nonhuman primates using several different regimens. In our laboratory we utilize the squirrel monkey (*Saimiri sciureus*), a New World monkey. We have developed a lesioning regimen that consists of either a series of two or six injections of MPTP at a concentration of 2.0 mg/kg (free-base) with 2 weeks between injections. With this lesioning regimen we avoid high animal mortality often experienced with MPTP use. Behavioral analysis using a clinical rating scale (CRS) we designed specifically for the MPTP-lesioned squirrel monkey, documents either mild (two injections) or moderate (six injections) parkinsonian motor behavior. Interestingly, over time, animals display motor recovery. By 6 weeks post-MPTP, animals show partial motor recovery and by 9 months post-MPTP both groups of monkeys show motor behavior that is indistinguishable from non-lesioned animals. Specifically, recovered animals no longer display akinesia or bradykinesia and show normal spontaneous movement (climbing and jumping), normal hand dexterity, and balance. Motor behavioral recovery has been observed in several other species of nonhuman primates including the marmoset, vervet, and macaque (Rose et al., 1989b; Rothblat and Schneider, 1994; Schneider et al., 1998; Elsworth et al., 2000). It has been shown that motor behavioral recovery takes place with the incomplete return of striatal dopamine. Neurotoxicant lesioning of the dopaminergic system in rodents, cats, and nonhuman primates suggest that there are pre- and post-synaptic alterations within remaining dopaminergic neurons and their targets, respectively, that may compensate for the deficient return of striatal dopamine and may account for motor behavioral recovery. Pre-synaptic indices include increased dopamine synthesis, turnover, and release, altered dopamine uptake, and sprouting of surviving dopaminergic neurons (Zigmond et al., 1990). Alterations in dopamine synthesis may be reflected by either an increase in the activity or total protein level of tyrosine hydroxylase (TH), the rate limiting enzyme of dopamine biosynthesis. In addition, alteration in dopamine uptake may reflect the level of dopamine transporter (DAT) protein because DAT is a key component in regulating dopamine synaptic occupancy (Gainetdinov et al., 2002). Post-synaptic indices include altered dopamine receptor number and function and their influence on neuropeptide expression, second messengers, transcription factors, and down-stream effector molecules especially within striatal medium-spiny neurons (Greengard et al., 1999; Gerfen, 2000; Vallone et al., 2000).

This study examines the relationship between motor behavioral recovery and pre- and post-synaptic indices of dopaminergic function, including striatal dopamine in the MPTP-lesioned squirrel monkey. We examined squirrel monkeys that were rendered either mildly or moderately parkinsonian and during early and late periods of recovery. Specifically, squirrel monkeys were administered MPTP as a series of either two or six injections 2 weeks between injections to generate either a mild or moderate parkinsonian group, respectively. Brain tissue was harvested at 6 weeks or 9 months after the last injection of MPTP. For neurochemistry and behavior, analyses were designed to compare individual groups. Molecular studies focused on the expression of two important pre-synaptic proteins, tyrosine hydroxylase and the dopamine transporter. As an initial approach to investigate post-synaptic changes, the pattern of expression of dopamine- and cAMP-responsive protein phosphatase of 32 kDa (DARPP-32), a molecule important for the down-stream action of dopamine neurotransmission in striatal medium spiny neurons, was examined. For molecular studies, analyses were designed to compare changes in the mild-lesioned group (two injections of MPTP) at the early and late time point, and changes in the moderate-lesioned group (six injections of MPTP) at the early and late time point.

## MATERIALS AND METHODS

### Animals

Twenty-four young–adult male squirrel monkeys (*Saimiri sciureus*) weighing 900–1,200 g each were used in these studies (Osage, St. Louis, MO). All procedures utilizing the nonhuman primate strictly followed guidelines set forth by the National Institutes for Health for the humane treatment of animals in research and had the approval of the University of Southern California Institutional Animal Care and Use Committee (IACUC). Animals were housed individually in a home cage. After quarantine, animals were acclimated to the facility for 30 days before behavioral analysis in their home cages.

### MPTP-Lesioning

MPTP (Sigma, St. Louis, MO) was administered in a series of subcutaneous injections set 2 weeks apart at a concentration of 2.0 mg/kg (free-base) dissolved in sterile water and made up fresh from a new 100-mg bottle each time. One group of animals ($n = 8$) received a series of two injections (for a total of 4.0 mg/kg MPTP, free-base). Another group of animals ($n = 8$) received a series of six injections of MPTP (for a total of 12.0 mg/kg, free-base). A saline injected group ($n = 8$) acted as control. Animals were divided into the following six groups with $n = 4$ per group: (1) saline-injected

**TABLE I. Parkinsonian Clinical Rating Scale Modified for Squirrel Monkey**

| Parkinsonian motor feature | Rating |
|---|---|
| 1. Spatial hypokinesia (movement around cage) | 0- Normal (uses entire cage space) |
| | 1- Utilizes most of the cage (at least 75% of cage space), but may be slow. |
| | 2- Definitely slowed, but uses more than 50% of cage space. |
| | 3- Definitely slowed, using less than 50% of cage space. |
| | 4- Does not move from a confined area, with little or no movement |
| 2. Body bradykinesia | 0- Normal movement around cage or bars |
| | 1- Slow or deliberate body movements, could be normal for age. |
| | 2- Moderately slow, intermittent limb dragging, moves without provocation. |
| | 3- Marked slowness, requires provocation to move arms or legs. |
| | 4- Frozen, little or no body movements regardless of provocation. |
| 3. Manual dexterity (right arm) | 0- Normal |
| | 1- Mildly slow or some loss of maneuverability of food items, could be normal for age. |
| | 2- Moderate slowness, noticeable effort needed to grab or maneuver food. |
| | 3- Marked slowness, with multiple attempts needed to grab food, may use both hands, may drop food. |
| | 4- Severe slowness, with inability to grab or maneuver food, may need to be hand fed. |
| 4. Manual dexterity (left arm) | 0- Normal |
| | 1- Mildly slow or some loss of maneuverability of food items, could be normal for age. |
| | 2- Moderate slowness, noticeable effort needed to grab or maneuver food. |
| | 3- Marked slowness, with multiple attempts needed to grab food, may use both hands, may drop food. |
| | 4- Severe slowness, with inability to grab or maneuver food, may need to be hand fed. |
| 5. Balance | 0- Normal |
| | 1- Slight tendency to hold on to cage, may be normal for age or no falls |
| | 2- Uses both hands intermittently for support or rare occasional falls |
| | 3- Uses both hands for support at all times or frequent falls |
| | 4- Continually hanging on for support or falls with any attempt to move |
| 6. Freezing (observation over 4 minute clinical evaluation) | 0- None, no freezing ever observed |
| | 1- Occasional mild (<5 sec) freezing episodes |
| | 2- Mild freezing episodes <5 sec duration or rare severe (>5 sec) |
| | 3- Frequent severe freezing observed >5 sec |
| | 4- Frozen most of the time |

harvested at 6 weeks; (2) saline-injected harvested at 9 months; (3) MPTP-injected 2-times harvested at 6 weeks after the last injection; (4) MPTP injected 2-times harvested at 9 months after the last injection; (5) MPTP-injected 6-times harvested at 6 weeks after the last injection; and (6) MPTP-injected 6-times harvested at 9 months after the last injection.

### Clinical Rating Scale for Motor Behavioral Analysis

Behavioral motor features were determined using a cage-side clinical rating scale (CRS) based on the Unified Parkinson's Disease Rating Scale (UPDRS) and modified for the squirrel monkey. Table I outlines the features of the clinical rating scale. The CRS consisted of six items including: (1) spatial hypokinesia (movement around cage); (2) body bradykinesia; (3) manual dexterity in left arm; (4) manual dexterity in right arm; (5) balance; and (6) freezing. Each item has a score of 0–4 resulting in a maximum motor score deficit of 24 points. A CRS score of <4 points is within the range of normal. Two investigators, blinded to the animal and treatment group, assessed motor features three times per week in the morning before feeding time. Baseline behavior was determined in the 2-week period before the first injection of MPTP or saline. Parkinsonian motor features using the CRS were first determined 2 weeks after the last injection of MPTP or saline, and continued on a weekly basis until tissue harvesting at 6 weeks or 9 months. Data for statistical analysis were carried out at baseline and post-injection time points of 2 weeks, 6 weeks, and 9 months. At each of these four time points a nonparametric Kruskal-Wallace analysis with Mann-Whitney post-hoc was carried out comparing saline, 2-time, and 6-time injected groups.

### Brain Tissue Harvest and Preparation

Brain tissue was harvested from four animals in each group at either 6 weeks or 9 months after the last injection of MPTP for a total of 24 animals. Animals were sedated with ketamine (0.3 ml of 100 mg/ml) followed by a lethal dose of sodium pentobarbital (2 ml of 50 mg/ml solution) and monitored for eye reflex, breathing, and heartbeat. On cessation of vital signs, brains were quickly removed, briefly cooled on wet ice, and sectioned at 3-mm thickness in the coronal plane using an acrylic brain block designed specifically for the squirrel monkey brain starting at a position approximately 3 mm rostral to the midbrain. This resulted in six rostral coronal slices of 3-mm thickness. One fresh slice through the midstriatum (at level A15–A12 in the anterior–posterior plane) of each animal was used to free-hand dissect out the entire caudate nucleus or putamen, sectioned into four pieces in the dorsal–ventral plane, and snap frozen on dry ice to be used for either high performance liquid chromatography (HPLC) analysis of dopamine and its metabolites or protein analysis using western immunoblotting (Emmers and Akert, 1963). The remaining caudal brain, which included the midbrain, was split at the mid-plane into two equal halves. Brain slices

were immersion fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) (pH 7.4) for 48 hr, cryoprotected in 20% sucrose in phosphate buffer (PB) (pH 7.4) for 24 hr, and frozen onto microscope slides using immersion into isopentane on dry ice. For immunohistochemical staining, tissue was cut at 30-μm thickness using a Leica 1950 cryostat. Sections were placed free-floating in PBS (pH 7.4) containing 0.01% thimerosal and refrigerated until used.

### HPLC Analysis of Dopamine and Its Metabolites

Neurotransmitter concentrations were determined according to an adaptation of Irwin et al. (1992) of the method of Kilpatrick et al. (1986). Tissues for analysis were homogenized in 0.4 N perchloric acid and centrifuged at 12,000 × *g* to separate precipitated protein. The protein pellet was resuspended in 0.5 N NaOH and the total protein concentration determined using the Coomassie Plus protein assay system (Pierce, Inc., Rockford, IL) using a Biotek Model Elx800 microplate reader and the software KCjunior. The concentrations of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), were assayed by HPLC with electrochemical detection (ECD). Samples were injected with an ESA autosampler. Dopamine and its metabolites were separated by a 150 mm × 3.2 mm reverse phase 3-μm diameter C-18 column (ESA, Chelmsford, MA) regulated at 28°C. The mobile phase MD-TM (from ESA) consisted of acetyl nitrile in PB and an ion-pairing agent delivered at a rate of 0.6 ml/min. The electrochemical detector was an ESA model Coularray 5600A with a 4-channel analytical cell with set potentials at −100 mV, 50 mV, and 220 mV. The HPLC was integrated with a Dell GX-280 computer with analytical programs including ESA Coularray for Windows software and the statistics package InStat (San Diego, CA). For neurochemistry, four separate basal ganglia regions were analyzed (ventral putamen, dorsal putamen, ventral caudate, or dorsal caudate) using multiple one-way ANOVA. Data were compared from the following six groups: (1) saline-injected harvested at 6 weeks; (2) saline-injected harvested at 9 months; (3) MPTP-injected 2-times harvested at 6 weeks after the last injection; (4) MPTP injected 2-times harvested at 9 months after the last injection; (5) MPTP-injected 6-times harvested at 6 weeks after the last injection; and (6) MPTP-injected 6-times harvested at 9 months after the last injection. Independent *t*-test showed no differences between the saline groups for each of the independent measures and as such, the two saline groups were collapsed into a single group for further analysis. Post-hoc contrasts with Bonferroni correction were carried out to determine the locus of any significant differences. For all statistical tests significance was set at $P < 0.05$.

### Stereologic Analysis of Nigrostriatal Neurons

To verify the degree of lesioning in the 2-time and 6-time injection groups at the time of harvest (9 months) the number of nigrostriatal dopaminergic neurons in the substantia nigra pars compacta (SNpc) was determined using unbiased stereology with the computer-imaging program BioQuant (BioQuant Image Analysis Inc., Nashville, TN) on a Dell computer and an Olympus BX-50 microscope equipped with a motorized stage and Retiga CCD camera. The total number of SNpc dopaminergic neurons was determined based on the method of Gunderson and Jensen (1987). The hemi-sectioned brain (total of six animals: two each from saline, 2-time, and 6-time MPTP-injected collected at 9 months after the last injection of MPTP) with its most rostral sections starting at level A9.0 based on the brain atlas for the squirrel monkey (Emmers and Akert, 1963) was mounted in the cryostat at a temperature of −18°C. Tissue was sliced at 30-μm thickness and every sixth section collected starting just before the rostral aspect of the substantia nigra (at level A8.0) completely through to the most caudal aspect when emergence of the pontine nucleus (at level A3.5) occurs. Tissue sections were stained for TH-immunoreactivity and counterstained for Nissl substance. The SNpc was delineated from the rest of the brain based on TH-immunoreactivity of remaining SNpc neurons because the MPTP-lesioning regimen of two or six injections used in these studies leads to 30–40% of remaining TH-immunoreactive dopaminergic neurons (in the case of the 6-time MPTP injections regimen) compared to saline (see Fig. 3). In addition, anatomic landmarks at the rostral and caudal aspects of the SNpc were also used for delineation including the cerebral peduncle, third nerve, and pontine nucleus as described in the atlas for the squirrel monkey (Emmers and Akert, 1963). Each stained ventral mesencephalon section was viewed at low magnification (10× objective) and the SNpc outlined and delineated from the ventral tegmental-immunoreactive neurons using the third nerve and cerebral peduncle as landmarks. Neurons were viewed at high magnification (80× objective) and counted if they displayed TH-immunoreactivity and had a clearly defined nucleus, cytoplasm, and nucleolus.

### Immunohistochemical Analysis of Striatal TH, DAT, and DARPP-32

The antibodies used in these studies were all purchased from Chemicon, Inc. (Temecula, CA) and included anti-tyrosine hydroxylase (polyclonal made in rabbit or monoclonal made in mouse), anti-dopamine transporter protein (monoclonal made in rat), and anti-DARPP-32 (polyclonal made in rabbit). Sections were rinsed in TBS (50 mM Tris pH 7.4 with 0.9% NaCl), exposed to primary antibody (concentration of 1:1,000) for 48 hr at 4°C, rinsed in TBS, and then exposed to secondary antibody made against the species of the primary antibody (ABC Elite Kit, Vectastain, Burlingame, CA). Antibody staining was visualized by development in diaminobenzoic acid/$H_2O_2$. To ensure that differences in staining intensity were due to differences in antigen expression, multiple sections from each treatment group and time point were carried out concurrently under identical staining conditions. Specificity of antibody probes was verified by methods that eliminated staining including: (1) omitting primary antibody; (2) omitting secondary antibody; or (3) omitting both primary and secondary antibodies. The relative intensity of labeling between stained sections in the different treatment groups was determined using computer assisted image analysis. Slides were simultaneously scanned on a high-resolution transmission light scanner (UMAX Powerlook 1120) and digitized images analyzed using NIH Image (version 1.66). Slide and section background (using a region of interest through the corpus cal-

losum) were captured. Because sections for comparison were stained simultaneously within an experiment, variability in background was minimal. Comparison of immunostaining based on the relative optical density was determined by analyzing images using a region of interest within the quadrant of the dorsal putamen (for DAT and DARPP-32 analysis). The dorsal putamen was selected for analysis because it plays a prominent role in motor behavior. At least six to eight sections from two or three animals were used for analysis. For statistical analysis, a one-way ANOVA comparing the individual groups was carried out. The saline groups were collapsed because an independent *t*-test showed no differences between the saline groups for each of the independent measures. Post-hoc contrasts with Bonferroni correction were carried out to determine the locus of any significant differences. For all statistical tests significance was set at $P < 0.05$.

### Western Immunoblot Analysis of Striatal TH and DAT

Western blotting was used to determine the relative protein expression of TH and DAT in the basal ganglia. The immunoblotting technique was described previously (Jakowec et al., 1995). Tissue dissected from the dorsal putamen was homogenized in buffer (25 mM Tris pH 7.4, 1 mM EDTA, 100 μM PMSF, 0.1% SDS). The dorsal putamen was selected for analysis because it plays a prominent role in motor behavior. Protein concentration was determined by the BCA method (Pierce, Inc., Rockford, IL). Equal amounts of protein (25 μg) were separated by the method of Laemmli (1970). Proteins were transferred to nitrocellulose filters by electroblotting in Towbin buffer (Towbin et al., 1979). Filters were blocked in TS-Blotto (50 mM Tris pH 7.4, 0.9% NaCl, 5% non-fat milk), then primary antibody (1:2,000), exposed to secondary antibody and visualized by chemiluminescence (Pierce, Inc.). Filters were apposed to film (Hyperfilm ECL, Amersham, Inc.) and processed in X-OMAT developer. Images were scanned into a computer using a Personal Laser Densitometer (Molecular Dynamics, Sunnyvale, CA) and the intensity of bands determined using computer assisted image analysis (NIH Image). The intensity of bands from Western blot autoradiographs was expressed as relative optical density with the saline injected group normalized to 100% because there was no significant difference between 6 weeks and 9 months. For statistical analysis, a one-way ANOVA comparing the individual groups was carried out. The saline groups were collapsed because an independent *t*-test showed no differences between the saline groups for each of the independent measures. Post-hoc contrasts with Bonferroni correction were carried out to determine the locus of any significant differences. For all statistical tests significance was set at $P < 0.05$.

## RESULTS

### Motor Behavioral Deficits After MPTP-Lesioning and the Time Course of Motor Behavioral Recovery

Motor behavior was assessed using a Clinical Rating Scale (CRS) as outlined in Table I and results shown in Figure 1. The baseline CRS scores before saline or MPTP administration for all groups of animals ranged from 1–2 of a total score of 24. Throughout the entire study, saline-injected animals had a CRS score of 1.2 ± 0.2. After completion of MPTP administration, all lesioned animals showed parkinsonian features including akinesia, bradykinesia, postural instability, and freezing. Two weeks after the last injection of MPTP, animals that received two injections of MPTP were mildly parkinsonian (8.3 ± 1.2), whereas animals that received six injections of MPTP were moderately parkinsonian (14.1 ± 1.2). The difference between the saline, 2-time, and 6-time MPTP-injected groups 2 weeks after the last injection of MPTP was significant At 6-weeks post-injection, there remained a significant differences between groups ($\chi^2 = 5.40$, $df = 2$, $P < 0.05$). The 6-time MPTP-injected group remained significantly more parkinsonian than the saline group (CRS of 6.1 ± 1.5 and 1.2 ± 0.2, respectively, $P < 0.014$). The 2-time MPTP-injected group was different than the saline group but did not reach significance (CRS of 4.9 ± 1.9 and 1.2 ± 0.2, respectively, $P < 0.2$). At the 9-month time point, both the 2-time and 6-time MPTP-injected groups displayed complete motor behavioral recovery (CRS score

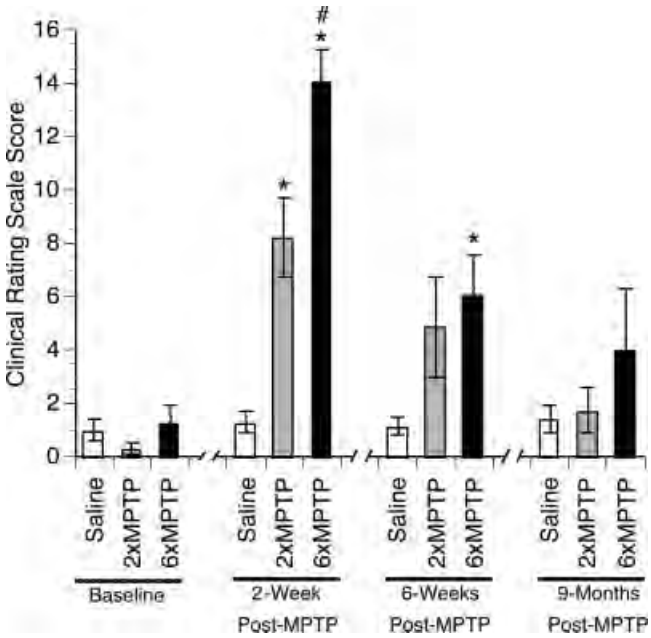


Fig. 1. Time course of motor behavior using the CRS. Baseline behavior was determined in the 2-week period before the first injection of MPTP or saline. Parkinsonian motor features using the CRS were first determined 2 weeks after the last injection of MPTP or saline, and continued on a weekly basis until tissue harvesting at 6 weeks or 9 months. Data for statistical analysis were carried out at baseline and post-injection time points of 2 weeks, 6 weeks, and 9 months. At each of these four time points a nonparametric Kruskal-Wallace analysis with Mann-Whitney post-hoc was carried out comparing saline, 2-times, and 6-times injected groups. *Indicates statistical significance compared to the saline CRS score at $P < 0.05$. #Indicates statistical significance compared to the 2-times MPTP-injected group CRS score at $P < 0.05$.

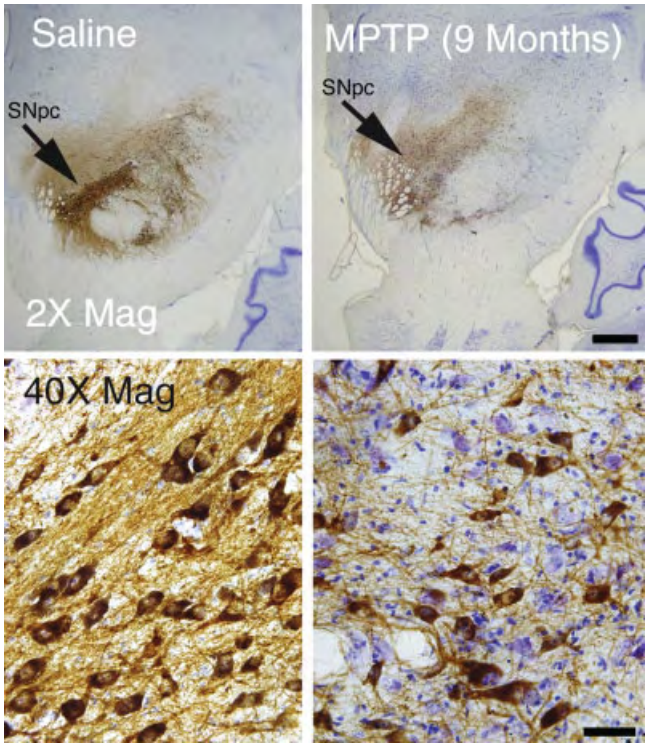


Fig. 2. Immunohistochemical staining for TH protein in the SNpc. Representative sections are shown through the mid-SNpc immunostained for TH and Nissl substance comparing saline-injected and 6-time MPTP-injected groups 9 months after the last injection. **Upper panels:** Photomicrographs at low magnification. **Lower panels:** Photomicrographs at high magnification. Note the severe depletion of TH-immunoreactive cells in the SNpc in the MPTP-lesioned SNpc despite the complete return of motor behavior. Scale bar = 800 μm (upper panels); 40 μm (lower panels). SNpc, substantia nigra pars compacta.

of 1.8 ± 0.9 and 4.0 ± 2.3, respectively) and were not significantly different from the saline group.

### Stereologic Counting of SNpc Dopaminergic Neurons

Unbiased stereologic counting methods were used to determine the degree of MPTP-lesioning based on number of TH-immunoreactive midbrain neurons at the 9-month time point. Saline animals had a total of 59,100 ± 4,440 TH-immunoreactive midbrain neurons, whereas the 2-time MPTP-injected group had 64.5% depletion (to 21,000 ± 2,500), and the 6-time MPTP-injected group had an 81% depletion in cell number (to 11,300 ± 900). Figure 2 is a representative image staining for TH and Nissl in the mid SNpc showing the large degree of dopaminergic cell loss even at 9 months after the last injection of MPTP in the 6-time injection group.

### HPLC Analysis of Dopamine and Its Metabolites

HPLC analysis of dopamine and its metabolites (HVA and DOPAC) was carried out on the dorsal putamen, dorsal caudate nucleus, ventral putamen, and ventral caudate nucleus of at least 3 animals in each group. The animal groups consisted of (1) saline control at 6 weeks and 9 months; (2) 2-times MPTP at 6 weeks; (3) 6-times MPTP at 6 weeks; (4) 2-times MPTP at 9 months; (5) 6-times MPTP at 9 months. Analysis was carried out to determine differences between all groups in the various anatomic regions. Because there was no significant difference between the saline groups at 6 weeks and 9 months for each anatomic region, these data were combined for analysis. Levels of dopamine and turnover ratio, defined as ([DOPAC + HVA]/dopamine) are shown in Table II and represented in Figure 3.

**Dopamine levels in the dorsal putamen.** Dopamine levels were significantly different in the dorsal putamen ($F = 23.2$, $df = 12$, $P < 0.001$). These data are represented in Figure 3A. Dopamine levels were significantly lower in the 2-time injected at 6 weeks (5.4 ± 5.2 ng dopamine/mg protein, 3.4% of control, $P < 0.001$), 6-time injected at 6 weeks (1.1 ± 0.3 ng dopamine/mg protein, 0.7% of saline control, $P < 0.001$), 2-time injected at 9 months (28.2 ± 11.1 ng dopamine/mg protein, 17.5% of saline control, $P < 0.001$), 6-time injected at 9 months (2.60 ± 0.3 ng dopamine/mg protein, 1.6% of saline control, $P < 0.001$) compared to saline (161.1 ± 18 mg ng dopamine/mg protein). There was no statistically significant difference between any MPTP-injected groups although there was a slight elevation of dopamine in the 2-time MPTP-injected group at 9 months that did not reach statistical significance.

**Dopamine turnover ratio in the dorsal putamen.** In the dorsal putamen, the dopamine turnover ratio was significantly different between groups ($F = 27.73$, $df = 12$, $P < 0.0001$). These data are represented in Figure 3B. The 2-time injected at 6 weeks (75.3 ± 37.7, $P < 0.0001$) and 6-time injected at 6 weeks (44.9 ± 6.7, $P < 0.01$) groups were significantly greater than saline control (1.2 ± 0.2). The turnover ratio in the 2-time MPTP-injected group was significantly higher than all other groups ($P < 0.01$).

**Dopamine levels in the ventral putamen.** Dopamine levels were different in the ventral putamen between groups ($F = 6.1$, $df = 12$, $P < 0.01$). These data are shown in Table I. These differences reached significance between the 2-time MPTP injected at 6 weeks (17.4 ± 16.9 ng dopamine/mg protein, 12.4% of saline control) compared to saline ($P < 0.05$) and the 6-time MPTP-injected group at 6 weeks (12.1 ± 4.5 ng dopamine/mg protein, 8.7% of saline control) compared to saline control ($P < 0.05$).

**Dopamine turnover ratio in the ventral putamen.** In the ventral putamen, the turnover ratio was different between groups ($F = 7.3$, $df = 12$, $P < 0.05$). These data are shown in Table I. This difference reached significance in the 2-time MPTP-injected group at 6 weeks (98.6 ± 62.5, $P < 0.05$) compared to saline control (1.6 ± 0.2) and all other groups.

**Dopamine levels in the dorsal caudate nucleus.** Dopamine levels were significantly different in the

**TABLE II. HPLC Analysis of Dopamine and its Metabolites**[†]

| Treatment | Time point | Region | Dopamine (ng/mg protein) | DOPAC (ng/mg protein) | HVA (ng/mg protein) | Turnover ratio |
|---|---|---|---|---|---|---|
| Saline | | Dorsal caudate ($n = 6$) | 95.0 ± 6.3 | 11.7 ± 1.7 | 81.6 ± 10.5 | 1.0 ± 0.1 |
| | | Ventral caudate ($n = 6$) | 133.6 ± 25.0 | 15.0 ± 1.2 | 121.7 ± 20.4 | 1.1 ± 0.1 |
| | | Dorsal putamen ($n = 6$) | 161.1 ± 18.1 | 23.5 ± 4.0 | 168.2 ± 15.6 | 1.2 ± 0.2** |
| | | Ventral putamen ($n = 6$) | 138.9 ± 25.4 | 22.0 ± 5.1 | 176.2 ± 13.9 | 1.6 ± 0.2** |
| 2 × MPTP | 6 weeks | Dorsal caudate ($n = 3$) | 6.6 ± 6.3* | 1.7 ± 1.5 | 29.3 ± 17.6 | 40.0 ± 21.5* |
| | | Ventral caudate ($n = 3$) | 16.6 ± 16.1* | 2.8 ± 2.2 | 29.3 ± 11.6 | 31.2 ± 18.6* |
| | | Dorsal putamen ($n = 3$) | 5.4 ± 5.2* | 1.2 ± 1.1 | 41.5 ± 19.8* | 75.3 ± 37.7* |
| | | Ventral putamen ($n = 3$) | 17.4 ± 16.9* | 3.4 ± 2.9 | 63.2 ± 14 | 98.6 ± 62.5* |
| | 9 months | Dorsal caudate ($n = 3$) | 24.1 ± 6.4* | 9.8 ± 3.5 | 61.6 ± 8.2 | 3.7 ± 1.5 |
| | | Ventral caudate ($n = 3$) | 68.6 ± 18.7 | 15.0 ± 5.7 | 88.9 ± 7.5 | 1.7 ± 0.4 |
| | | Dorsal putamen ($n = 3$) | 28.2 ± 11.1* | 8.5 ± 3.8 | 102.8 ± 24.7 | 5.6 ± 2.3** |
| | | Ventral putamen ($n = 3$) | 83.9 ± 32.2 | 14.3 ± 4.0 | 174.4 ± 31.2 | 2.8 ± 0.7** |
| 6 × MPTP | 6 weeks | Dorsal caudate ($n = 3$) | 0.6 ± 0.2* | 0.5 ± 0.1 | 22.7 ± 3.5 | 52.3 ± 20.3* |
| | | Ventral caudate ($n = 3$) | 5.9 ± 2.8* | 2.4 ± 0.4 | 36.9 ± 2.6 | 17.0 ± 11.9 |
| | | Dorsal putamen ($n = 3$) | 1.1 ± 3* | 0.9 ± 0.3 | 42.5 ± 11.8* | 44.9 ± 6.7*,** |
| | | Ventral putamen ($n = 3$) | 12.1 ± 4.5* | 6.8 ± 2.9 | 98.9 ± 17.2 | 11.5 ± 3.9** |
| | 9 months | Dorsal caudate ($n = 3$) | 8.0 ± 4.0* | 2.6 ± 0.7 | 73.4 ± 29.5 | 20.9 ± 12 |
| | | Ventral caudate ($n = 3$) | 31.5 ± 13.9 | 10.2 ± 2.3 | 89.2 ± 26.0 | 5.4 ± 2.5 |
| | | Dorsal putamen ($n = 3$) | 2.6 ± 27* | 2.12 ± .07 | 55.7 ± 10.3 | 23.2 ± 5.7** |
| | | Ventral putamen ($n = 3$) | 35.8 ± 14.2 | 11.8 ± 0.82 | 71.3 ± 25.6 | 4.8 ± 1.8** |

[†]Specific anatomical regions (dorsal caudate, ventral caudate, dorsal putamen, or ventral putamen) from individual groups were analyzed using a one-way ANOVA with a Bonferroni post-hoc analysis.

*Significantly different from the saline group ($P < 0.05$).

**Significantly different from the 2 × MPTP/6 weeks group ($P < 0.05$).

dorsal caudate nucleus ($F = 66.5$, $df = 12$, $P < 0.001$). These data are represented in Figure 3C. Dopamine levels were significantly lower in the 2-time injected at 6 weeks (6.6 ± 6.3 ng dopamine/mg protein, 6.9% of control, $P < 0.001$), 6-time injected at 6 weeks (0.6 ± 0.2 ng dopamine/mg protein, 0.6% of saline control, $P < 0.001$), 2-time injected at 9 months (24.1 ± 6.4 ng dopamine/mg protein, 25.4% of saline control, $P < 0.001$), 6-time injected at 9 months (8.0 ± 4.0 ng dopamine/mg protein, 8.4% of saline control, $P < 0.001$) compared to saline (95.0 ± 6.3 mg ng dopamine/mg protein). There was no statistically significant difference between any MPTP-injected groups although there was a slight elevation of dopamine in the 2-time MPTP-injected group at 9 months, which did not reach statistical significance.

**Dopamine turnover ratio in the dorsal caudate nucleus.** In the dorsal caudate nucleus, the dopamine turnover ratio was significantly different between groups ($F = 5.8$, $df = 12$, $P < 0.05$). These data are represented in Figure 3D. The 2-time injected at 6 weeks (40.0 ± 21.5, $P < 0.05$) and 6-time injected at 6 weeks (52.3 ± 20.3, $P < 0.05$) groups were significantly greater than saline control (1.0 ± 0.1).

**Dopamine levels in the ventral caudate nucleus.** Dopamine levels were different in the ventral caudate nucleus between groups ($F = 6.1$, $df = 12$, $P < 0.02$). These data are shown in Table I. These differences reached significance between the 2-time MPTP injected at 6 weeks (16.6 ± 16.1 ng dopamine/mg protein, 12.4% of saline control, $P < 0.05$) and the 6-time MPTP injected at 6 weeks (5.9 ± 2.8 ng dopamine/mg protein, 4.4% of saline control, $P < 0.05$) compared to saline (133.6 ± 25.0 ng dopamine/mg protein).

**Dopamine turnover ratio in the ventral caudate nucleus.** In the ventral caudate, the dopamine turnover ratio was significantly different between groups ($F = 4.7$, $df = 12$, $P < 0.05$). These data are shown in Table I. The 2-time injected at 6 weeks (31.2 ± 18.6, $P < 0.05$) was significantly different from saline control (1.1 ± 0.1).

## Analysis of TH Protein Expression

The pattern of expression of TH protein in the caudate nucleus and putamen of animals from all groups was determined using both immunocytochemistry and western immunoblotting (Fig. 4). In saline injected animals, TH-immunoreactivity (TH-ir) at the level of the mid-striatum showed intense staining throughout the entire caudate nucleus and putamen as seen in coronal sections at low magnification (Fig. 4A). At high magnification the staining appeared as a dense fibrous network with staining in both puncta and dark fibers of various thicknesses (Fig. 4B). At 6 weeks after the last MPTP injection, tissues from the 2-time and 6-time injected groups showed severe depletion of TH-ir. Remaining TH-ir appeared as light background staining with dark staining TH-ir fibers. The TH-ir fibers were most apparent in the 2-time compared to the 6-time injected groups (compare Fig. 4D and 4H). Examination of TH-ir at 9 months after the last injection of MPTP showed a

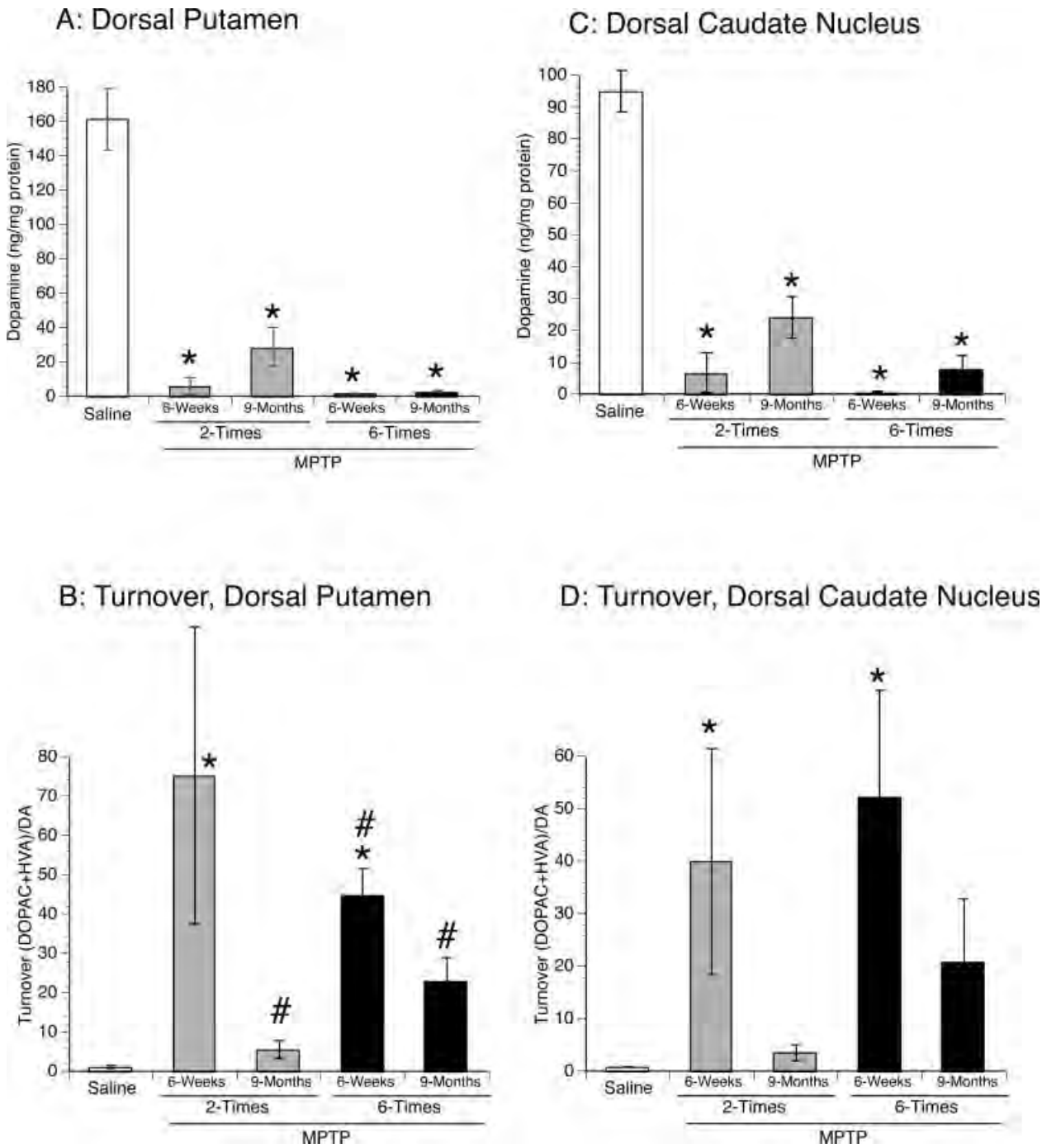


Fig. 3. Neurochemical analysis of dopamine and its metabolites. Data are shown for the analysis of dopamine from (**A**) the dorsal putamen and (**C**) the dorsal caudate nucleus from at least three animals per group except saline where $N = 6$. Saline groups consist of pooled data from tissues collected at both the 6-week and 9-month time points because statistical analysis showed no difference. Tissue for analysis was collected from the 2-time and 6-time MPTP-injected groups at either 6 weeks or 9 months after the last injection of MPTP. Dopamine turnover defined as (DOPAC + HVA)/DA, is depicted in (**B**) for the dorsal putamen and (**D**) the dorsal caudate nucleus. *Indicates statistical significance compared to saline ($P < 0.05$). #Indicates statistical significance compared to the 2-time MPTP-injected group at 6 weeks ($P < 0.05$).

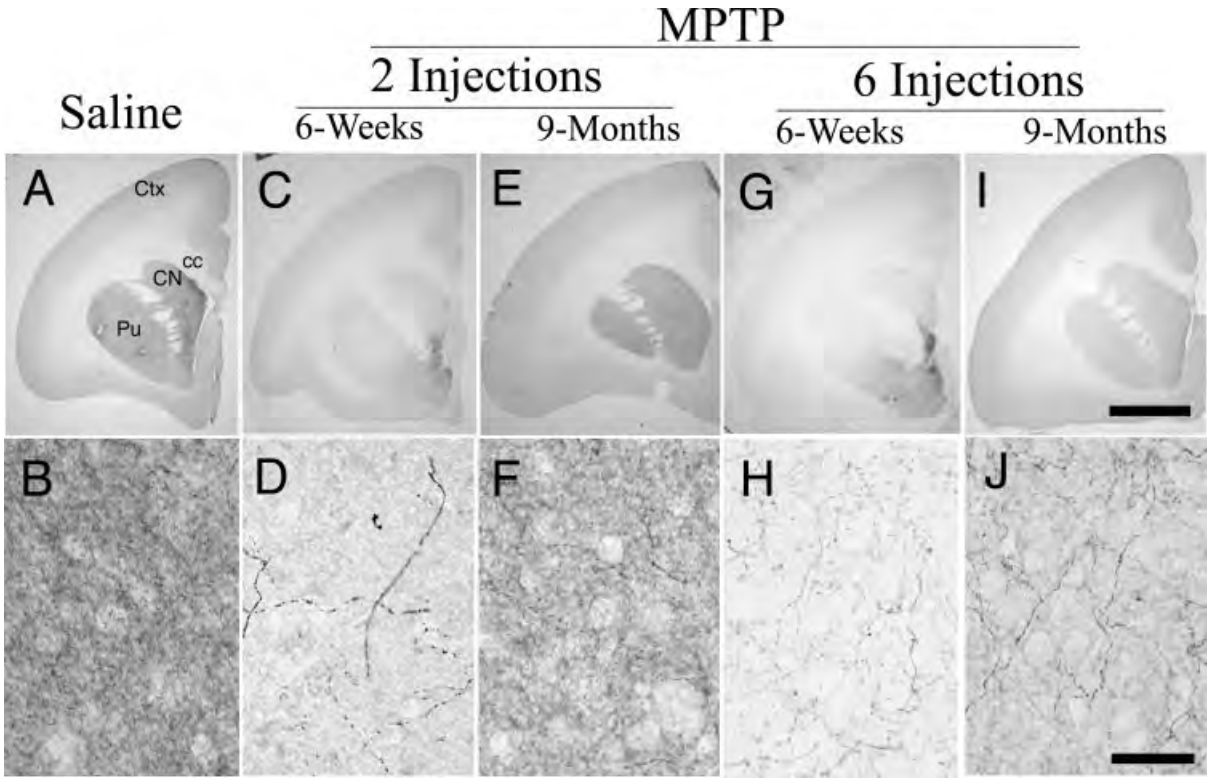


Fig. 4. Immunohistochemical staining for TH protein in the caudate nucleus and putamen. **Upper panels:** Representative images at low magnification of hemi-sections in the coronal plane at the level of the mid-striatum at the anterior–posterior plane between 14–15 mm. **Lower panels:** High magnification images obtained from the most dorsal quadrant of the caudate nucleus. **A:** TH-ir is seen as dark immuno-staining throughout the caudate nucleus and putamen. **B:** At higher magnification dark TH-ir appears as a thick fibrous network. **C:** At 6-weeks after two injections of MPTP there is a significant reduction in TH-ir throughout the caudate nucleus and putamen and (**D**) only a light fibrous TH-ir network remains. **E:** At 9 months after the last of the 2-time MPTP injections TH-ir increases throughout the striatum and (**F**) an increase in the fibrous network is observed. **G:** At 6 weeks after the last injection in the 6-time MPTP regimen results in a severe depletion of TH-ir (**H**), which appears as very light staining with a small number of distinct fibers remaining. **I:** At 9 months after the last of the series of six injections of MPTP there is an increase in the degree of TH-ir (**J**) corresponding to an increase in the degree of TH-ir fiber density. CN, caudate nucleus; Pu, putamen; cc, corpus callosum; ctx, cortex. Scale bar = 0.5 mm (I, images in the top row); 15 μm (J, images in the bottom row).

partial return of TH-ir in both the 2-time (compare Fig. 4C and 4E) and 6-time injected groups (compare Fig. 4G and 4I). The degree of TH-ir return was greater in the 2-time injected group (compare Fig. 4E and 4I) with the 2-time injected group showing a greater intensity of TH-ir (compare Fig. 4F and 4J), in the amount of fibers and neuropil background.

Western immunoblotting analysis was carried out to determine the relative degree of TH protein expression in the dorsal putamen from animals in all groups. A representative immunoblot is shown in Figure 5. There was no difference in the level of TH protein in saline animals harvested at 6 weeks or 9 months. Therefore, the results from the saline animals were pooled. The relative amount of TH protein was significantly lower compared to saline control in the 2-time injected at 6 weeks (16.2 ± 0.6% of saline control, $P < 0.01$), the 2-time injected at 9 months (31.8 ± 1.7% of saline control, $P < 0.01$), 6-time injected at 6 weeks (8.9 ± 1.1% of saline control, $P < 0.01$), and the 6-time injected at 9 months (20.7 ± 1.7% of saline control, $P < 0.01$). The relative amount of TH protein in the 2-time MPTP injected at 9-months and the 6-time MPTP injected at 9 months showed a slight increase compared to the other MPTP-injected groups. This increase, however, did not reach significance.

### Analysis of DAT Protein Expression

The pattern of expression of DAT protein in the caudate nucleus and putamen from animals in all groups was determined using immunohistochemistry (Fig. 6). In saline injected animals, DAT-immunoreactivity (DAT-ir) in coronal sections at the level of the mid-striatum showed intense staining throughout as shown at low magnification (Fig. 6A). At high magnification the staining appeared as a dense fibrous network similar to that seen with TH-ir with staining in both puncta and dark fibers of various thicknesses (Fig. 6B). Tissues from

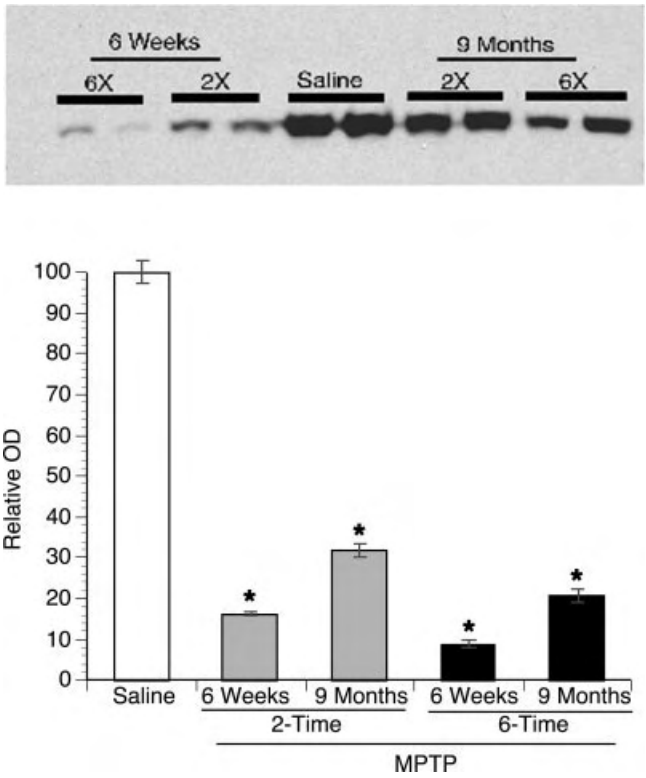


Fig. 5. Western immunoblot for TH protein in the caudate nucleus. **Upper panel:** Representative immunoblot comparing the degree of TH protein expression in the caudate nucleus from tissues collected from the 2-time and 6-time MPTP-injected groups at 6 weeks and 9 months after the last injection. Note the highest amount of expression in the saline group, depletion at 6 weeks in both MPTP-lesioned groups and partial return in both MPTP-lesion groups at 9 months. **Lower panel:** Relative optical density (OD) of immunoblot data derived from two samples from three different animals from each group. *MPTP-lesioned groups statistically significant from saline ($P < 0.05$).

both the 2-time and 6-time MPTP injection groups at 6 weeks post-injection of MPTP showed significant depletion of DAT-ir. Remaining DAT-ir appeared as a light background staining with dark staining fibers. The degree of DAT-ir was greater in the 2-time injection group compared to the 6-time injected group (compare Fig. 6D and 6H). At 9 months after the last injection of MPTP, analysis of DAT-ir showed a partial return in both the 2-time injected (compare Fig. 6C and 6E) and 6-time injected groups (compare Fig. 6G and 6I). The degree of DAT-ir return was greater in the 2-time injected group (compare Fig. 6E and 6I) with the 2-time injected group showing a greater intensity of DAT-ir (compare Fig. 6F and 6J).

Western immunoblotting analysis was carried out to determine the relative degree of DAT protein expression in the dorsal putamen from animals in all groups. There was no difference in the level of DAT protein in saline animals harvested at 6 weeks or 9 months. Therefore, the results from the saline animals were pooled. The relative amount of DAT protein was significantly lower compared to saline control in the 2-time injected at 6 weeks (13.0 ± 1.3% of saline control, $P < 0.05$), 2-time injected at 9 months (43.1 ± 2.8% of saline control. $P < 0.05$), 6-time injected at 6 weeks (15.9 ± 3.2% of saline control, $P < 0.05$), and 6-time injected at 9 months (32.6 ± 5.0% of saline control, $P < 0.05$). DAT expression was greater significantly, however, in the 2-time injected at 9 months and the 6-time injected at 9 months compared to the MPTP-injected groups at 6 weeks ($P < 0.05$).

### Analysis of DARPP-32 Protein Expression

The pattern of expression of DARPP-32 protein in coronal sections of the mid-caudate nucleus and putamen of animals from all groups was determined using immunocytochemistry (Fig. 7). The highest degree of immunoreactivity was seen in cell bodies throughout the striatum, with some neuropil staining. After MPTP administration, the 2-time injected at 6 weeks and the 6-time injected at 6 weeks showed reduction in the intensity of cell body and neuropil staining. The degree of immunoreactivity returned to levels that were similar to that of the saline control groups in the 2-time injected at 9 months and 6-time injected at 9 months.

The relative optical density of DARPP-32 protein immunoreactivity was carried out in the dorsal region of these sections from all groups (Fig. 7). Optical density is reported as a % relative to saline. There was no difference in the optical density of DARPP-32 protein in saline animals harvested at 6 weeks or 9 months. Therefore, the results from the saline animals were pooled. The optical density of DARPP-32 protein was significantly lower in the 2-time injected at 6 weeks (76.4 ± 3.7% of saline) and the 6-time injected at 6 weeks (63.2 ± 2.5% of saline) compared to saline ($P < 0.001$). However the 2-time injected at 9 months and the 6-time injected at 9 months were not significantly different from the saline group (101.1 ± 1.9% and 91.9 ± 3.7%, respectively).

## DISCUSSION

The systemic administration of the neurotoxicant MPTP in the nonhuman primate squirrel monkey (*Saimiri sciureus*) leads to the selective destruction of dopaminergic neurons in the substantia nigra pars compacta and ventral tegmental area and the depletion of dopamine in the caudate nucleus and putamen (Burns et al., 1983; Langston et al., 1984). In addition to the neurochemical changes, MPTP-lesioned nonhuman primates display profound deficits in motor behavior that resemble features of parkinsonism including akinesia, bradykinesia (slowness), postural instability (balance), and freezing (Petzinger and Langston, 1998). Similar to patients with idiopathic PD, MPTP-lesioned animals respond to dopamine replacement therapy (levodopa [L-DOPA] + carbidopa) with improvement of motor features and also display L-

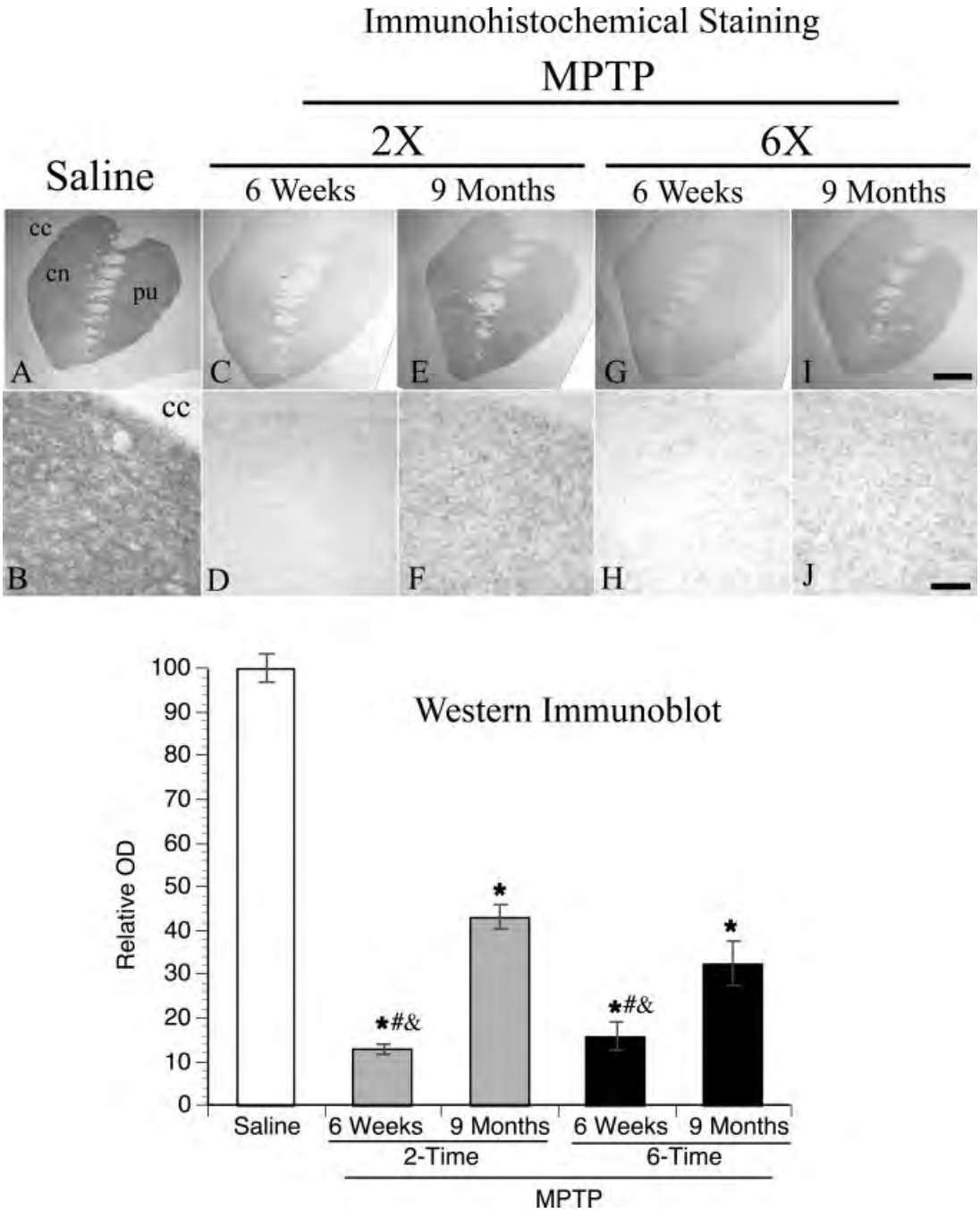


Fig. 6. Immunohistochemical staining for dopamine transporter in the caudate nucleus and putamen. **Upper panel:** Series of photomicrographs images shows coronal sections through the mid-striatum at level in the anterior–posterior plane at 14–16 mm were stained for DAT-ir and representative sections photographed at low magnification (12.5× in upper panels; scale bar = 0.2 mm) and at high magnification (400× in lower panels; scale bar = 6 μm). **A:** Sections from saline injected animals showed intense dark staining throughout the caudate nucleus and putamen and (**B**) appeared as a dark fibrous network with a high background. **C:** At 6 weeks after the last injection in the 2-time MPTP regimen DAT-ir was significantly reduced with (**D**) a small number of dark DAT-ir fibers remaining on a lightly immunoreactive background. **E:** After 9 months the 2-time MPTP injection regimen showed the return of DAT-ir that (**F**) appeared as a fibrous network of various sizes with increased DAT-ir background. **G:** At 6 weeks after the regimen of six injections of MPTP DAT-ir was reduced significantly throughout the caudate nucleus and putamen with (**H**) a small number of DAT-ir fibers remaining. **I:** At 9 months after the regimen of six injections of MPTP there was a slight return of DAT-ir that (**J**) appeared as an increase in the staining of fibers of various sizes resulting in increased background staining. **Lower panel:** Relative optical density for Western immunoblot analysis of DAT expression in the dorsal putamen. *Indicates statistical significance compared to the saline control group ($P < 0.05$). #Indicates statistical significance compared to the 2-time MPTP-injected group at the 9-month time point ($P < 0.05$). &Indicates statistical significance compared to the 6-time MPTP-injected group at the 9-month time point ($P < 0.05$).

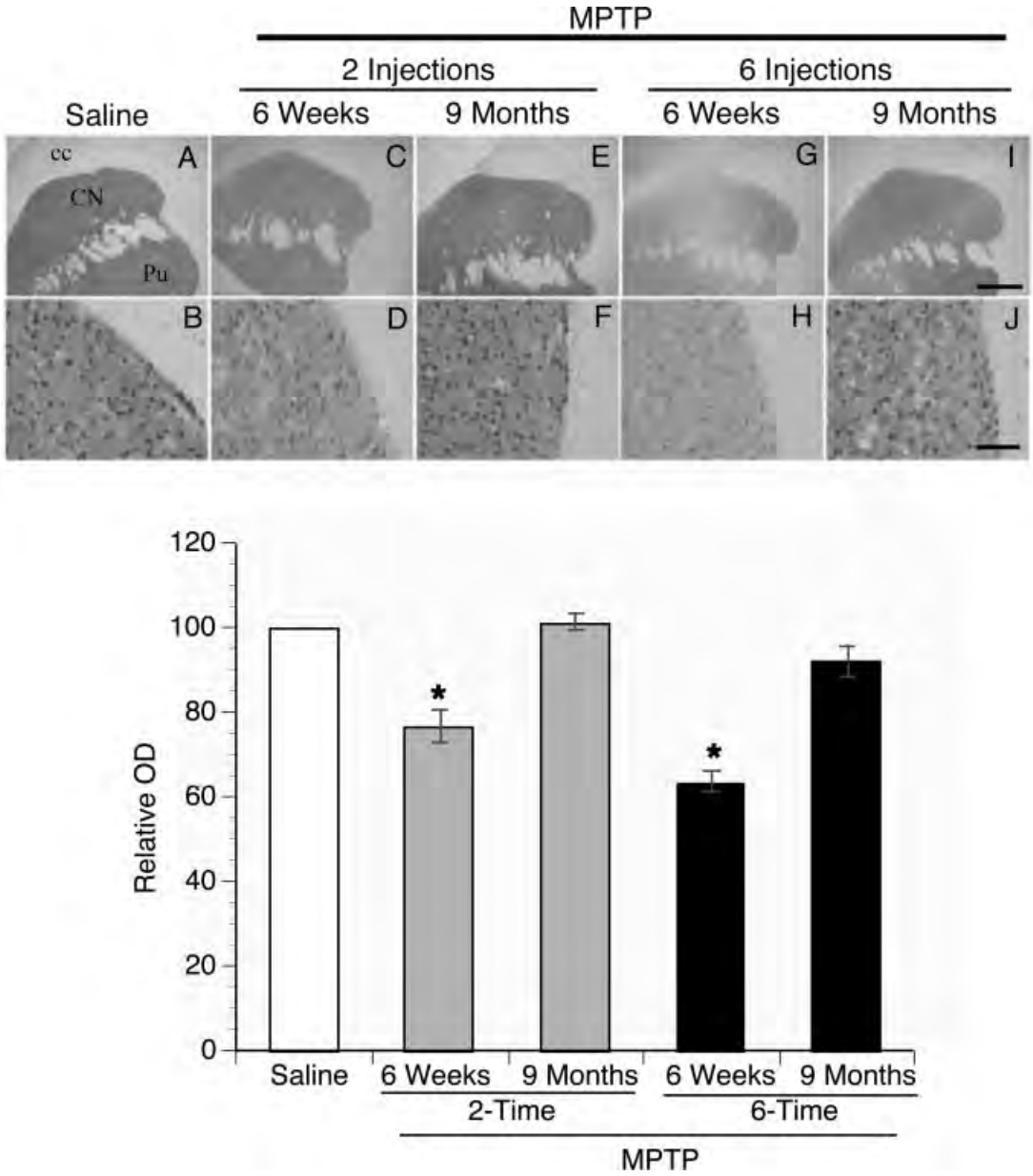


Fig. 7. Immunohistochemical staining for DARPP-32 protein in the caudate nucleus and putamen. **Upper panel:** Series of photomicrographs images shows coronal sections through the mid-striatum were stained for DARPP-32 immunoreactivity and representative sections photographed at low magnification (12.5× in the upper panels; scale bar = 0.25 mm) and at high magnification in a region representing the dorsal putamen adjacent to the corpus callosum (400× in the lower panels; scale bar = 7 μm). **A:** Saline animals showed immunoreactivity throughout the striatum at low magnification, (**B**) which appeared as staining predominantly within cell nuclei and to a lesser extent in the neuropil. At 6 weeks after MPTP-lesioning the 2-time and 6-time injected animals showed a reduction in the intensity of overall immunoreactivity (**C,G**), which appeared as reduced staining within both cell bodies themselves and to a lesser degree the neuropil (**D,H**). Immunostaining was less intense in the 6-time injected group compared to the 2-time injected group (D,H). At 9 months after MPTP-lesioning immuno-staining in the 2-time injection group was similar to that seen in saline animals (**E,F**). Immuno-staining at 9 months after MPTP-lesioning in the 6-time injection group had immuno-staining (I and J) that was less intense then both the saline and 2-time injected animals at the same time point. **Lower panel:** Relative optical density of DARPP-32 immuno-staining in the dorsal putamen in all treatment groups ($n$ = 8 sections/group). *Indicates statistical significance compared to the saline control group ($P < 0.05$). CN, caudate nucleus; Pu, putamen; cc, corpus callosum.

DOPA-related motor complications, including dyskinesia (Schneider, 1989; Boyce et al., 1990a,b; Petzinger et al., 2001).

In our study, as in others, the degree of motor behavioral deficits is dependent on the severity of MPTP lesioning because monkeys administered two injections of MPTP (2.0 mg/kg, free-base per injection) have mild symptoms whereas animals receiving a series of six injections of MPTP display more severe parkinsonism. Additionally, we observed that motor recovery was apparent by 6 weeks post-MPTP and was complete in mild and severely lesioned animals by 9 months, with animals appearing indistinguishable from normal non-lesioned animals. Fully recovered animals display normal spontaneous movements (jumping and climbing throughout cage), as well as normal hand dexterity and balance. Behavioral recovery has been reported in several species of nonhuman primates after MPTP-lesioning (Rose et al., 1989a,b; Elsworth et al., 1990; Kurlan et al., 1991; Elsworth et al., 2000). Interestingly, the reported degree of recovery is variable and may be dependent on several factors including the time after lesioning, the species, and the mode of behavioral assessment. For example, studies in the MPTP-lesioned marmoset showed recovery with respect to gross akinesia by 3–4 months post-lesioning but ''lack of spontaneous movement and poor coordination of activities'' were still evident and persisted at 18 months post-MPTP-lesioning (Rose et al., 1989a,b; Ueki et al., 1989).

Using our MPTP lesioning regimen, we saw significant dopamine depletion in the caudate nucleus and putamen at 6 weeks post-lesioning that persisted at 9 months despite motor behavioral recovery. This degree of depletion, however, did show regional differences (dorsal versus ventral caudate nucleus and putamen). For example, at 6 weeks after the last injection of MPTP dopamine levels in the dorsal putamen and dorsal caudate nucleus were depleted >93% compared to dopamine levels in saline control. At 9 months after the last injection of MPTP, when full motor recovery was observed, dopamine levels in the dorsal putamen and dorsal caudate nucleus remained low. This was in contrast to both the ventral putamen and ventral caudate nucleus where the degree of depletion was less than that seen in the respective dorsal regions at 6 weeks and the return of dopamine at 9 months after MPTP-lesioning was greater. Our findings are similar to other nonhuman primate studies that have reported reversal of parkinsonian features despite persistent dopamine loss (Rose et al., 1989a; Schneider and Rothblat, 1991; Gnanalingham et al., 1995; Rothblat and Schneider 1995). These studies also reported greater return of dopamine in the ventral striatum, the greatest extent measured in the nucleus accumbens, the most ventral aspect of the striatum (Rose et al., 1989b; Gnanalingham et al., 1995). Analogous to the MPTP-lesioned nonhuman primate, studies reporting behavioral recovery despite extensive dopamine depletion have been made in the MPTP-lesioned cat model examining the reversal of akinesia, bradykinesia, and sensorimotor neglect and in the 6-OHDA-lsioned rat examining the reversal of sensorimotor neglect, aphagia, adipsia, and bradykinesia (Ungerstedt, 1971; Zigmond and Stricker, 1973; Marshall and Teitelbaum, 1974; Marshall and Gotthelf, 1979; Schneider et al., 1994).

Pre- and post-synaptic mechanisms may play a role in compensating for the incomplete return of dopamine, and may underlie behavioral recovery after neurotoxicant injury to the basal ganglia. The pre-synaptic mechanisms include increased dopamine biosynthesis due to altered affinity of TH for its cofactors, increased expression of TH protein, increased dopamine release, and decreased synaptic dopamine uptake. These pre-synaptic adaptations in remaining dopaminergic neurons are thought to lead to normalization of extracellular levels of dopamine especially in animals with lesions resulting in <80% depletion of total striatal intracellular and extracellular dopamine pools (Robinson and Wishaw, 1988; Zhang et al., 1988; Altar and Marien 1989; Abercrombie et al., 1990; Castaneda et al., 1990). Animals with >80% total dopamine depletion show only partial normalization of extracellular striatal dopamine (Castaneda et al., 1990). The relative contribution of post-synaptic changes in targets of dopaminergic neurons to behavioral recovery is thought to be dependent on the extent of dopamine depletion within the extracellular space (Zigmond et al., 1984, 1990; Zhang et al., 1988). These post-synaptic changes include altered dopamine receptor number and function and their influence on the expression of neuropeptides, second messengers, transcription factors, and down-stream effector molecules, especially within striatal medium-spiny neurons (Creese et al., 1977; Zigmond et al., 1984).

In the MPTP-lesioned squirrel monkey, we observed an increase in dopamine metabolites relative to dopamine, defined as the turnover ratio ([DOPAC + HVA]/dopamine), during the early motor behavioral recovery period (at 6 weeks). Turnover ratio showed regional differences within the striatum and was highest in the dorsal putamen and dorsal caudate nucleus. Consistent with our study, others have reported elevated turnover ratio highest in those areas of the striatum (dorsal region) with the greatest amount of dopamine depletion (Onn et al., 1986; Schneider and Rothblat, 1991; Elsworth et al., 2000). At 9 months after MPTP-lesioning when motor behavioral recovery was complete, however, turnover ratio was diminished in all regions of the striatum compared to the 6-week time point. This observation of a decline in turnover ratio during motor recovery has also been observed in the MPTP marmoset (Gnanalingham et al., 1995). Changes in turnover ratio is thought to be due to changes in the rate and firing of dopaminergic neurons or increase in dopamine release from surviving dopaminergic neurons (Hollerman and Grace, 1990). The result of elevated turnover ratio is to attempt to maintain extracellular levels of dopamine.

The level of extracellular dopamine is also influenced by the DAT, a protein localized to nigrostriatal terminals (Gainetdinov et al., 2002). Decreased DAT

expression that was observed after MPTP-lesioning may lead to increased synaptic levels of dopamine and increase in the diffusion of dopamine to depleted regions (Schneider et al., 1994; Rothblat and Schneider, 1999) during the early period of motor behavioral recovery in the squirrel monkey. At 9 months, when motor behavioral recovery was complete, DAT expression increased throughout the striatum, even in the dorsal striatum where dopamine levels remained persistently low. An increased DAT expression is likely due to the presence of sprouting in surviving nigrostriatal dopaminergic neurons (Finkelstein et al., 2000; Jakowec et al., 2004). Similar to striatal dopamine and its metabolites, there were regional differences in DAT expression, with the greatest return noted in the ventral striatum (data not shown). The return of striatal DAT during motor recovery seems to vary among studies and may depend on the degree and method of lesioning, animal species, neurotoxicant (MPTP vs. 6-OHDA), and the time point of analysis. Most studies suggest that sprouting may rely on some degree of surviving dopaminergic neurons to serve as a template, because partial lesioning shows more robust expression of markers for sprouting in comparison to near-complete lesioning (Frohna et al., 1997; Mitsumoto et al., 1998; Finkelstein et al., 2000).

Tyrosine hydroxylase is an enzyme important in the biosynthesis of dopamine and is often considered a marker of nigrostriatal neuron sprouting (Bezard et al., 2000; Rothblat et al., 2001; Jakowec et al., 2004). At 6 weeks post-MPTP lesioning, when animals are displaying early motor behavioral recovery, dopamine levels are low and TH-immunoreactivity is severely depleted. At 9 months when animals are recovered fully, however, TH expression shows partial return. This alteration in TH is analogous to DAT, and may also be an indicator of neuronal sprouting. Interestingly, increased TH expression was observed within the dorsal putamen, despite persistently low dopamine levels (up to 98% depletion). Similar to the conclusions of others, our study suggests that sprouting of TH immunoreactive fibers may precede the biosynthesis and return of significant levels of striatal dopamine (Jakowec et al., 2004; Mitsumoto et al., 1998).

One post-synaptic marker of the functional integrity of striatal medium spiny neurons is DARPP-32. DARPP-32 is a down-stream effector molecule that regulates a number of enzymatic and receptor mediated cellular processes that are essential for normal basal ganglia function (Ouimet et al., 1984; Anderson and Reiner, 1991; Nishi et al., 1997). The activity of DARPP-32 can be regulated by its phosphorylated state as well as expression levels, which may be influenced by dopaminergic or glutamatergic neurotransmission (Greengard et al., 1999; Nishi et al., 2005). Most studies on DARPP-32 expression have examined its phosphorylation state shortly after injury and not total protein levels (Raisman-Vozari et al., 1990). In our study, the relative DARPP-32 protein levels, as indicated by a pan-specific antibody, were measured by optical density of striatal immunostained tissues. DARPP-32 immunostaining was initially reduced in cells during the early period of behavioral recovery (6 weeks post-MPTP) in the 2-time and 6-time MPTP-injected groups. However, by 9 months and after motor behavioral recovery, DARPP-32 immunostaining had fully returned despite regional areas of persistent and marked dopamine depletion. Given the dichotomy between the return of DARPP-32 protein expression and continued dopamine depletion suggests that other non-dopaminergic systems, such as the glutamatergic, may make a more significant contribution to the function of medium spiny neurons during motor behavioral recovery in the MPTP-lesioned squirrel monkey (Greengard et al., 1999; Wang et al., 2004; Nishi et al., 2005). We are currently investigating this hypothesis in our lab through the administration of specific glutamate receptor antagonists.

In conclusion, our study indicates that several months after MPTP-lesioning, squirrel monkeys display full motor behavioral recovery using a clinical rating scale that measures parkinsonian features. Interestingly, the return of striatal dopamine appears incomplete with more pronounced return in the ventral striatum in comparison to the dorsal striatum. This finding is consistent with other reports using neurotoxicant-lesioning models of the basal ganglia in both rodents and other species of nonhuman primates. Elevated dopamine turnover ratio and decreased DAT expression seem to be two pre-synaptic mechanisms that may be important for the compensation of dopamine depletion in early behavioral recovery. The increase of both TH and DAT expression during the time when motor behavioral recovery is complete suggests neuronal sprouting as an important mechanism in late stage recovery even within dopamine depleted regions. In addition, post-synaptic mechanisms within the medium spiny neurons, as indicated by altered DARPP-32 expression, seem to be potentially important targets for investigation.

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Neurobiology of Disease

# Effects of Treadmill Exercise on Dopaminergic Transmission in the 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine-Lesioned Mouse Model of Basal Ganglia Injury

**Giselle M. Petzinger,[1,3] John P. Walsh,[2] Garnik Akopian,[2] Elizabeth Hogg,[1] Avery Abernathy,[1] Pablo Arevalo,[1] Patty Turnquist,[3] Marta Vučković,[1] Beth E. Fisher,[3] Daniel M. Togasaki,[1] and Michael W. Jakowec[1,3]**

[1]Department of Neurology, [2]Andrus Gerontology Center, and [3]Department of Biokinesiology and Physical Therapy, The George and MaryLou Boone Center for Parkinson's Disease Research, University of Southern California, Los Angeles, California, 90033

**Studies have suggested that there are beneficial effects of exercise in patients with Parkinson's disease, but the underlying molecular mechanisms responsible for these effects are poorly understood. Studies in rodent models provide a means to examine the effects of exercise on dopaminergic neurotransmission. Using intensive treadmill exercise, we determined changes in striatal dopamine in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned mouse. C57BL/6J mice were divided into four groups: (1) saline, (2) saline plus exercise, (3) MPTP, and (4) MPTP plus exercise. Exercise was started 5 d after MPTP lesioning and continued for 28 d. Treadmill running improved motor velocity in both exercise groups. All exercised animals also showed increased latency to fall (improved balance) using the accelerating rotarod compared with nonexercised mice. Using HPLC, we found no difference in striatal dopamine tissue levels between MPTP plus exercise compared with MPTP mice. There was an increase detected in saline plus exercise mice. Analyses using fast-scan cyclic voltammetry showed increased stimulus-evoked release and a decrease in decay of dopamine in the dorsal striatum of MPTP plus exercise mice only. Immunohistochemical staining analysis of striatal tyrosine hydroxylase and dopamine transporter proteins showed decreased expression in MPTP plus exercise mice compared with MPTP mice. There were no differences in mRNA transcript expression in midbrain dopaminergic neurons between these two groups. However, there was diminished transcript expression in saline plus exercise compared with saline mice. Our findings suggest that the benefits of treadmill exercise on motor performance may be accompanied by changes in dopaminergic neurotransmission that are different in the injured (MPTP-lesioned) compared with the noninjured (saline) nigrostriatal system.**

*Key words: in situ* **hybridization; neurochemistry; neuroplasticity; substantia nigra; Parkinson's disease; tyrosine hydroxylase**

## Introduction

Recent studies from several laboratories including ours have shown that exercise can have a beneficial effect in patients with Parkinson's disease (PD) and in rodent models of PD (Comella et al., 1994; Tillerson et al., 2001, 2003; Bezard et al., 2003; Fisher et al., 2004; Chen et al., 2005; Faherty et al., 2005). In both the 6-hydroxydopamine (6-OHDA) lesioned rat and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesioned mouse, exercise initiated either before or during neurotoxicant exposure has been shown to be neuroprotective, as demonstrated by the attenuation of striatal dopamine loss. Our previous studies have shown that high-intensity treadmill exercise, initiated 5 d after administration of MPTP, a period when neurotoxicant-induced cell death is completed, can also lead to improved motor performance in the MPTP lesioned mouse (Jackson-Lewis et al., 1995; Fisher et al., 2004). In addition, we found downregulation of the dopamine transporter (DAT), a protein important in regulating the uptake of dopamine, and upregulation of dopamine receptor D2, a receptor whose activation is important in eliciting motor behavior. Given the critical role of dopamine in motor learning and execution, and our interest in neuroplasticity of the injured basal ganglia, in this study we have examined the effects of exercise on other potential compensatory changes of the striatal dopaminergic system including total striatal dopamine levels and release. We further examined whether exercise-induced changes in proteins important in dopamine biosynthesis (tyrosine hydroxylase, TH) and uptake (DAT) may be accompanied by changes in their respective mRNA transcript expression within midbrain dopaminergic neurons and whether exercise leads to changes in the number of substantia nigra neurons.

For these studies, we used four groups of mice including (1) saline, (2) saline plus exercise, (3) MPTP, and (4) MPTP plus exercise. Intensive treadmill running was initiated 5 d after MPTP

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Correspondence should be addressed to Dr. Michael W. Jakowec, Department of Neurology, University of Southern California, 1333 San Pablo Street, MCA-241, Los Angeles, CA 90033. E-mail: mjakowec@surgery.usc.edu.

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lesioning and continued for 28 d of running (5 d/week). Brain tissue was harvested from all four groups at the completion of exercise. Analysis included (1) measurement of striatal dopamine and its metabolites by HLPC and (2) striatal dopamine release using fast-scan cyclic voltammetry in slice cultures. Unbiased stereological counting of nigrostriatal dopaminergic neurons was used to determine any differences in cell numbers between groups. Our studies indicate that exercise may have a differential effect on the dopaminergic system in MPTP- versus saline-treated mice. In the MPTP-lesioned mice, exercise had an effect on dopamine release, which was region specific, but not in total striatal dopamine levels. In saline-treated mice, exercise increased total striatal dopamine levels without a significant effect on dopamine release. In addition, in our study, we determined that MPTP administration led to 50% cell loss, with no difference in substantia nigra cell numbers between MPTP and MPTP plus exercise mice. Downregulation of DAT and TH transcript expression was detected in saline plus exercise-treated mice only.

## Materials and Methods

*Animals.* Mice used for these studies were young adult (8–10 weeks old) male C57BL/6J mice supplied from The Jackson Laboratory (Bar Harbor, ME). There were four treatment groups including the following: (1) saline, (2) saline plus exercise, (3) MPTP, and (4) MPTP plus exercise. Animals were housed five to a cage and acclimated to a 12 h shift in light/dark cycle so that the exercise occurred during the animals normal wake period. All experiments were performed in accordance with the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals* (NIH publication 80-23, revised 1996) and approved by the University of Southern California Institutional Animal Care and Use Committee.

*MPTP lesioning.* MPTP (Sigma, St. Louis, MO) was administered in a series of four intraperitoneal injections of 20 mg/kg (free base) at 2 h intervals for a total administration of 80 mg/kg. This regimen leads to ~60% loss of nigrostriatal neurons, as determined by unbiased stereological techniques for both TH staining and Nissl substance and an 80–90% depletion of striatal dopamine levels (Jackson-Lewis et al., 1995; Jakowec et al., 2004). Nigrostriatal cell loss is complete by day 3 after MPTP administration as determined by counting remaining nigrostriatal TH immunoreactive cells and reduced silver staining for degenerating neurons (Jackson-Lewis et al., 1995; Jakowec et al., 2004).

*Treadmill exercise.* One week before the start of the treadmill exercise paradigm, sixty mice that could maintain a forward position on the 45 cm treadmill belt for 5 min at 5.0 m/min were randomly assigned to the four groups to ensure that all animals performed similarly on the treadmill task before MPTP lesioning. The treadmill used in these studies was a model EXER-6M treadmill manufactured by Columbus Instruments (Columbus, OH). A non-noxious stimulus (metal-beaded curtain) was used as a tactile incentive to prevent animals from drifting back on the treadmill. As a result, shock-plate incentive was not used and stress related to the activity was minimized. Exercise was initiated 5 d after saline or MPTP lesioning when cell death is complete. Mice from each of the two exercise groups (saline plus exercise and MPTP plus exercise) were run at the same time in the six-lane treadmill. Exercise duration was incrementally increased starting with 30 min on day 1 to reach the goal duration of two sessions of 30 min each (for a total of 60 min), 5 d/week (with a 5 min warm-up period), for a total of 28 d of exercise (corresponding to a final of 42 d after MPTP lesioning). Treadmill speed and exercise duration for each group was increased when all mice within each group maintained a forward position on the treadmill belt for 75% of the running period. To control for any nonexercise effects of treadmill running (handling, novel environment, noise, and vibration), nonexercised groups were placed on the top of the treadmill apparatus for a time period equivalent to exercise training (Fukai et al., 2000; Kojda et al., 2001).

*Motor behavior analysis with the rotarod.* An accelerating rotarod (five-lane accelerating rotarod; Ugo Basile, Comerio, Italy) was used to measure motor balance and coordination during the treadmill exercise period. The rotarod consisted of a rotating spindle (diameter, 3 cm) where mice were challenged for both speed and alternating rotational direction. Velocity of the rod was set to 30 rpm with changes in direction (forward-reverse) every 24 s. Each mouse was placed in a separate compartment on the rotating rod and the latency to fall was automatically recorded by magnetic trip plates. All groups of mice were tested once per week. The first week was recorded 3 d after starting the treadmill exercise. The test consisted of five consecutive trials each separated by 1 min, with a maximum cutoff latency of 200 s. Data were subsequently collected for each mouse once per week until completion of the exercise paradigm (corresponding to week 6).

*Tissue collection.* Brain tissue from all groups of mice was collected on the last day of exercise (day 28 of exercise corresponding to 42 d after MPTP lesioning). Striatal brain tissue was also collected from a subset of animals from each experimental group at 5 d of exercise (10 d postlesioning) to determine the degree of dopamine depletion at an earlier exercise and MPTP time point. Mice were killed by cervical dislocation for fresh tissues (for HPLC, fast-scan cyclic voltammetry, and *in situ* hybridization histochemistry) or by pentobarbital followed by transcardial perfusion with fixative (for immunohistochemistry and unbiased stereological counting). Striatal tissues for HPLC analysis were collected fresh en bloc corresponding to anatomical regions from bregma 1.20 to bregma 0.60, with borders dorsal to the anterior commissure, ventral to the corpus callosum, medial to the lateral ventricle, and 2.5 mm lateral from midline, and frozen until analysis. *In situ* hybridization histochemistry, fast-scan voltammetry, and immunohistochemistry were performed on coronal sections corresponding to bregma 1.30 to bregma 0.00. In addition, to evaluate the initial degree of MPTP-mediated striatal dopamine depletion, brain tissue for HPLC analysis was collected at 10 d post-MPTP lesioning from a subset of nonexercise mice from both the saline and MPTP groups.

*HPLC analysis of dopamine and its metabolites.* Neurotransmitter concentrations were determined according to an adaptation of Irwin et al. (1992) from the method of Kilpatrick et al. (1986). Tissues for analysis were homogenized in 0.4 N perchloric acid and centrifuged at 12,000 × *g* to separate precipitated protein. The protein pellet was resuspended in 0.5 N NaOH and the total protein concentration determined using the Coomassie Plus protein assay system (Pierce, Rockford, IL) using a Biotek Model Elx800 microplate reader (Biotek Instruments Wincoski, VT) and KCjunior software. The concentrations of dopamine, 3,4-dihydroxyphenylacetic (DOPAC), and homovanillic acid (HVA) were assayed by HPLC with electrochemical detection. Samples were injected with an ESA (Chelmsford, MA) autosampler. Dopamine and its metabolites were separated by a 150 × 3.2 mm reverse phase 3-$\mu$m-diameter C-18 column (ESA) regulated at 28°C. The mobile phase MD-TM (ESA) consisted of acetylnitrile in phosphate buffer and an ion-pairing agent delivered at a rate of 0.6 ml/min. The electrochemical detector was an ESA model Coularray 5600A with a four-channel analytical cell with three set potentials at −100, 50, and 220 mV. The HPLC was integrated with a Dell GX-280 computer with analytical programs including ESA Coularray for Windows software and the statistics package InStat (GraphPad Software, San Diego, CA).

*Measurement of dopamine release by fast-scan cyclic voltammetry in brain slices.* Fast-scan cyclic voltammetry was used for the analysis of dopamine release from coronal, *in vitro* brain slices of the striatum (Patel and Rice, 2006). Brains were removed and placed in cooled (1–4°C), modified, and oxygenated artificial CSF (aCSF) containing the following (in mM): 124 NaCl, 1.3 $MgSO_4$, 3.0 KCl, 1.25 $NaH_2PO_4$, 26 $NaHCO_3$, 2.4 $CaCl_2$, 10.0 glucose, equilibrated with a 95% $O_2$/5% $CO_2$ mixture to obtain a pH value of 7.3–7.4. In the modified aCSF, some sodium was replaced with sucrose to reduce tissue excitability during brain slice cutting (sucrose 124 mM, NaCl 62 mM) to maintain the osmotic balance of normal aCSF. Hemicoronal striatal slices were cut from the rostral end of the tissue at a thickness of 400 $\mu$m with a Vibratome 1000 (Vibratome, St. Louis, MO). Slices were immediately placed in oxygenated aCSF and were slowly brought to room temperature (23°C). Slices remained in solution for 2 h before and throughout all recording sessions. Single slices were transferred to the recording chamber (Haas ramp style gas interface

chamber) and bathed continuously with the oxygenated aCSF solution maintained at a temperature of 32°C. Disc carbon fiber microelectrodes (CFMEs) were made from 7 mm unsized carbon fiber (Goodfellow Corporation, Devon, PA) by electrophoretic anodic deposition of paint (Schulte and Chow, 1996). Extracellular dopamine was monitored at the CFME every 100 ms by applying a triangular waveform (−0.4 to +1.0 V vs Ag/AgCl, 300 V/s). Currents were recorded with a modified VA-10× Voltammetric and Amperometric Amplifier (NPI Electronic, Tamm, Germany). Data acquisition was controlled by Clampex 7.0 software (Molecular Devices, Menlo Park, CA). Electrical stimulation was used to elicit dopamine efflux with a twisted, bipolar, nichrome electrode placed on the surface of the slice. Single pulses (0.1 ms, 200 $\mu$A) were generated with a Master-8 pulse generator (AMPI, Jerusalem, Israel). Constant current of 200 $\mu$A and 0.1 ms duration were obtained by using an A360R Constant Current Stimulus Isolator (World Precision Instruments, Sarasota, FL). Stimulus intervals between pulses were not <5 min. The CFMEs were inserted 75–100 $\mu$m into the slice at a position 100–200 $\mu$m from the stimulating electrode pair (Miles et al., 2002). Each slice was sampled for dopamine at five sites, which represented medial to lateral and dorsal to ventral dimensions. Three rostral slices were examined in each mouse and the values were averaged for each animal. Changes in extracellular dopamine were determined by monitoring the current over a 200 mV window at the peak oxidation potential for dopamine. Background-subtracted cyclic voltammograms were created by subtracting the current obtained before stimulation from the current obtained in the presence of dopamine. To convert oxidation current to dopamine concentration, electrodes were calibrated with dopamine standard solutions after experimental use. The mechanism of evoked dopamine release in our voltammetry experiments was tested in a set of brain slices by stimulating striatal tissue using the following sequence of solutions changes: control aCSF containing 2.4 mM $Ca^{2+}$ and 1.3 mM $Mg^{2+}$ followed by a reduced $Ca^{2+}$ aCSF solution containing 0.5 mM $Ca^{2+}$ and 3.2 mM $Mg^{2+}$, followed by washout with the control aCSF solution and then ending in a control aCSF solution containing 1 $\mu$M tetrototoxin (TTX).

The kinetics of the dopamine signal evoked by intrastriatal stimulation was studied by monitoring the cyclic voltammetry signal for 1 s before and 5 s after intrastriatal stimulation at a sampling rate of once every 100 ms (10 Hz). The decay of the dopamine signal was determined by normalizing postpeak dopamine measurements to the peak dopamine measured. The decay constant was then determined from a single exponential fit of the decay in dopamine signal according to the following equation: $y = Ae^{-kt}$, where $A$ is the peak dopamine signal at time 0 and the constant $-k$ is the decay rate for exponential decay of the dopamine signal. ANOVAs were performed between all groups for the decay rate constant ($-k$) (Mosharov and Sulzer, 2005).

*Immunohistochemical staining.* The relative expression of striatal TH immunoreactivity (TH-ir) and DAT immunoreactivity were determined in tissues sections using commercially available primary antibodies, including rabbit polyclonal anti-TH and mouse monoclonal anti-DAT (Millipore, Bedford, MA) using a validated approached to compare relative patterns of expression as described by Burke et al. (1990). Primary antibody binding was visualized using fluorescently labeled secondary antibodies with AlexaFluor 680 or AlexaFluor 800 (Licor, Lincoln, NE). To ensure that differences in staining intensity were because of differences in antigen expression, multiple sections from each of the different treatment groups were handled in identical staining conditions concurrently. Control experiments excluding either primary antibody or secondary antibody were also performed to verify staining specificity. For image analysis, three or four animals per treatment group and 10–12 sections per animal were captured at low magnification and digitized. The relative optical density (expressed as arbitrary units within the linear range of detection) of the dorsal lateral striatum was determined by subtracting the relative optical density of the corpus callosum as background.

*Unbiased stereological counting of dopaminergic neurons.* The number of nigrostriatal dopaminergic neurons in the substantia nigra pars compacta (SNpc) was determined using unbiased stereology with the computer-imaging program BioQuant Nova Prime (BioQuant Imaging, Nashville, TN) and an Olympus BX-50 microscope (Olympus Optical, Tokyo, Japan) equipped with a motorized stage and digital Retiga-cooled CCD camera (Q-Imaging, Burnaby, British Columbia, Canada). Brain tissue was prepared from three mice in each group. Tissue was sliced at 30 $\mu$m thickness and every sixth section collected and stained for TH-ir using a rabbit polyclonal antibody (Millipore) and counterstained for Nissl substance (Jakowec et al., 2004; Petzinger et al., 2005). The SNpc was delineated from the rest of the brain based on TH-ir. Section collection started rostral to the substantia nigra at bregma −2.50 mm before the closure of the third ventricle through to the prominence of the pontine nuclei at bregma −4.24 mm according to the stereotaxic atlas of the mouse brain (Paxinos and Franklin, 2001). Each stained ventral mesencephalon section was viewed at low magnification (10× objective) and the SNpc outlined and delineated from the ventral tegmental-immunoreactive neurons using the third nerve and cerebral peduncle as landmarks. Neurons were viewed at high magnification (80× objective) and counted if they displayed TH-ir and had a clearly defined nucleus, cytoplasm, and nucleolus. The total number of SNpc dopaminergic neurons was determined based on the method of Gundersen and Jensen (1987).

In situ *hybridization histochemistry.* Brains for *in situ* hybridization were quickly removed and frozen in isopentane on dry ice, and tissues were processed as described previously (Jakowec et al., 1995, 2004). Selected slides were dipped in NTB-2 (Kodak, Rochester, NY) photographic emulsion, developed in D-19 developer, and counter stained with cresyl violet. To minimize potential sources of variation between different experiments, slides that were to be compared were processed in the same experiment using identical hybridization buffers, probe concentration, probe preparation, wash regimen, and emulsion exposure. Images of midbrain cells were captured using an Olympus BX-51 microscope and the computerized image analysis program BioQuant (BioQuant Imaging). Emulsion grains were counted above individual neurons within the SNpc if they displayed a stained cytoplasm and evident nucleus.

*Statistical analysis of data.* Statistical analysis was performed using SPSS version 14.0 for Windows (SPSS, Chicago, IL) or InStat software (GraphPad Software). Differences in behavioral tests between groups were analyzed using repeated-measures ANOVA with the between-subjects factors being lesion (saline or MPTP) and intervention (exercise or no exercise) and the within subject factor being time. For HPLC analysis, immunocytochemistry staining, and grain counting for *in situ* hybridization histochemistry, a two-way ANOVA was performed to compare the different groups and examine for significant interactions. *Post hoc* contrasts with Bonferroni correction were performed to determine the locus of any significant differences. The data from the studies of dopamine release using fast-scan cyclic voltammetry were analyzed using repeated-measures ANOVA with the between-subjects factors being lesion (saline or MPTP) and intervention (exercise or no exercise) and the within-subjects factor being electrode location (positions 1–5). *Post hoc* testing was performed in those cases where warranted. Within-subject effects were performed using the Huynh-Feldt correction for sphericity. For all analyses, a significance level of $p < 0.05$ was used.

## Results

The time course of improvement in running velocity of both the saline plus exercise and MPTP plus exercise groups over the 28 d of treadmill running is shown in Figure 1. Saline plus exercise mice at day 1 started at 13.3 m/min and the MPTP plus exercise mice at day 1 started at 7.6 m/min. In the first week of treadmill running, the saline group increased velocity to 14 ± 1.4 m/min that further increased to 22.6 ± 0.3 m/min by the final week. The MPTP plus exercise group had a running velocity that increased to 9.2 ± 1.1 m/min during the first week that further increased to 20.5 ± 0.7 m/min in the last week. As shown in our previous study, *post hoc* analysis demonstrated a significant difference in velocity at day 1 between the saline plus exercise and MPTP plus exercise groups and this difference was not significant at the completion of the treadmill running regimen (Fisher et al., 2004). The

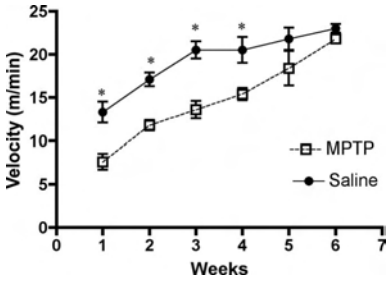


**Figure 1.** Analysis of motor behavior on motorized treadmill. Both saline and MPTP-lesioned mice were run on the motorized treadmill for 28 d (5 d per week). The running velocity of the mice in each group ($n = 12$) were determined three times per week and compared. The graph demonstrates that (1) MPTP-lesioned mice had a running velocity less than the saline group, where the asterisk indicates significant difference ( $p < 0.05$), and (2) both saline- and MPTP-lesioned groups improved in running velocity, and by the final 2 weeks, the difference between the two groups was not significant ( *post hoc* analysis; $p < 0.05$; *t* test). Error bars indicate SEM.

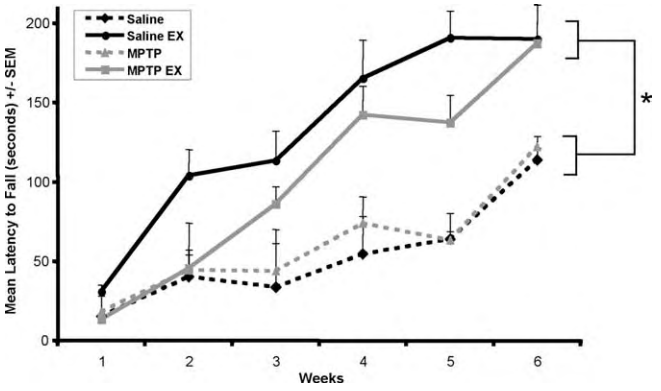


**Figure 2.** Analysis of behavior on rotarod mice from all groups were tested once a week for latency to fall from an accelerating rotarod (mean and SEM in seconds per week). There was a significant effect of exercise (treadmill running) on the mean latency to fall (in seconds) from the accelerating rotarod, where the asterisk represents significant differnce between the exercise and no exercise groups ($F_{(3,44)} = 9.587$; $p < 0.0001$). Both MPTP plus exercise and saline plus exercise mice performed better on the rotarod compared with the nonexercised groups. There was no significant effect of MPTP on mean latency to fall ($F_{(3,44)} = 0.851$; $p = 0.504$) and no significant interaction between exercise and MPTP on mean latency to fall ($F_{(3,44)} = 0.965$; $p = 0.435$).

running velocity of the MPTP nonexercise mice at the final day of treadmill exercise was similar to the velocity of the MPTP plus exercise group at day 1 of the exercise regimen (data not shown) (Fisher et al., 2004).

As a second measure of motor performance, mice from all four groups were tested for their latency to fall from the accelerating rotarod (Fig. 2). Because of the high degree of challenge of this task, mice from all groups initially performed poorly. We observed overtime, however, that there was a significant effect of exercise (treadmill running) on the mean latency to fall (in seconds) from the accelerating rotarod ($F_{(3,44)} = 9.587$; $p < 0.0001$). Both MPTP plus exercise and saline plus exercise mice performed better on the rotarod compared with the nonexercised groups. There was no significant effect of MPTP on mean latency to fall ($F_{(3,44)} = 0.851$; $p = 0.504$) and no significant interaction between exercise and MPTP on mean latency to fall ($F_{(3,44)} = 0.965$; $p = 0.435$).

HPLC analysis was used to determine levels of striatal dopamine, its metabolites HVA and DOPAC, and the metabolites turnover ratio, defined as follows: [(DOPAC + HVA)/dopamine]. These data are shown in Table 1 and Figure 3. First, in the 5 d exercise group, there was a significant effect of MPTP on dopamine levels ($F_{(3,16)} = 39.52$; $p < 0.0001$) and dopamine turnover ($F_{(3,16)} = 88.30$; $p < 0.0001$). MPTP induced dopamine depletion in the MPTP plus no exercise group (48.0 ± 8.4 ng dopamine/mg protein) compared with saline plus no exercise group (269.5 ± 24.9 ng dopamine/ng protein), which corresponded to 82% depletion. The MPTP induced dopamine depletion in the MPTP plus exercise group (4.9 ± 0.2 ng dopamine/mg protein) compared with saline plus exercise group (285.1 ± 30.3 ng dopamine/ng protein), which corresponded to 98% depletion. There were no significant differences in striatal dopamine levels between exercise and no exercise animals in either the MPTP- or saline-treated animals. However, there was a significant decrease in turnover ratio in the MPTP plus exercise compared with the MPTP plus no exercise groups only ($F_{(3,16)} = 8.30$; $p < 0.02$)

HPLC analysis of striatal dopamine at the completion of the 28 d of treadmill running showed that there was a significant effect of MPTP on the degree of dopamine depletion ($F_{(3,16)} = 229.3$; $p < 0.0001$). MPTP induced dopamine depletion in the MPTP plus no exercise group (77.9 ± 12.0 ng dopamine/mg protein) compared with saline plus no exercise group (246.9 ± 19.8 ng dopamine/ng protein), which corresponded to 68% depletion. The MPTP induced dopamine depletion in the MPTP plus exercise group (69.8 ± 11.7 ng dopamine/mg protein) compared with saline plus exercise group (315.2 ± 9.0 ng dopamine/ng protein), which corresponded to 78% depletion. In addition, there was a significant interaction between lesioned state and exercise on striatal dopamine levels ($F_{(3,16)} = 7.78$; $p = 0.015$). This interaction was because of a significant effect of exercise on the saline-treated group, in which saline plus exercise mice had a higher level of striatal dopamine compared with saline mice. There was no significant difference in striatal dopamine levels comparing MPTP plus exercise with MPTP plus no exercise mice. There were no significant effects of MPTP or exercise, or interaction between these two factors on turnover ratio, with ratios of 0.36 for MPTP, 0.34 for MPTP plus exercise, 0.26 for saline, and 0.34 for the saline plus exercise group.

Dopamine release induced by intrastriatal stimulation was determined using fast-scan cyclic voltammetry at completion of the 28 d of treadmill running. Coronal slices at the level of midstriatum were sampled with electrodes placed at five sites, as illustrated in Figure 4. For measurements in the contralateral striatum, a mirror-image placement was used. Measurements were made using four to six mice from each group and from four slices per mouse. For each animal, a single mean value was determined at each electrode location by averaging all data collected. Stimulation-induced dopamine release varied with the location of the electrode ($F_{(3,20)} = 9.211$; $p < 0.001$) in both saline and MPTP-treated mice. MPTP lesioning dramatically decreased stimulation-induced dopamine release compared with saline-treated mice ($F_{(3,20)} = 26.450$; $p < 0.001$). Exercise had a significant effect in the MPTP-lesioned mouse that was dependent on electrode location ($F_{(3,20)} = 2.875$; $p = 0.039$). Additional analysis using *post hoc t* tests demonstrated that there was significant dopamine release at electrode positions three ( $p = 0.036$) and four ( $p = 0.023$) but not electrode positions one ( $p = 0.723$), two ( $p = 0.252$), and five ( $p = 0.141$) in MPTP plus exercise mice compared with MPTP plus no exercise mice. Exercise did not have an effect in the unlesioned animals (no overall effect, $F_{(3,20)} = 0.084$, $p = 0.781$; no interaction between exercise and striatal location, $F_{(3,20)} = 2.586$, $p = 0.088$), but it did have an effect on lesioned animals, depending on the location in the stri-

**Table 1. HPLC analysis of striatal dopamine and its metabolites**

| | | | Dopamine | DOPAC | HVA | Turnover |
|---|---|---|---|---|---|---|
| Exercise day 5 | No exercise | Saline | 269.5 ± 24.9 | 33.4 ± 4.5 | 33.1 ± 3.2 | 0.3 ± 0.08 |
| | | MPTP | 48.0 ± 8.4* | 11.7 ± 1.9* | 87.2 ± 9.6 | 2.1 ± 0.28* |
| | Exercise | Saline | 285.1 ± 30.3 | 36.2 ± 1.8 | 26.3 ± 2.2 | 0.2 ± 0.06 |
| | | MPTP | 4.9 ± 0.2* | 1.4 ± 0.1* | 4.7 ± 0.1# | 1.2 ± 0.14*,# |
| Exercise day 28 | No exercise | Saline | 246.9 ± 19.8 | 36.5 ± 4.5 | 27.4 ± 1.7 | 0.3 ± 0.01 |
| | | MPTP | 77.9 ± 12.0* | 14.7 ± 2.4* | 13.5 ± 1.9* | 0.4 ± 0.01 |
| | Exercise | Saline | 315.2 ± 9.0+ | 39.3 ± 2.8 | 25.9 ± 1.3 | 0.2 ± 0.01 |
| | | MPTP | 69.8 ± 11.7* | 11.3 ± 1.5* | 13.5 ± 1.2* | 0.4 ± 0.05 |

The concentration of dopamine, DOPAC, and HVA and turnover ratio were analyzed in each experimental group ($n = 6$ per group) at 5 d of exercise (corresponding to 10 d postlesioning) and also at 28 d of exercise (corresponding to 42 d postlesioning). The turnover ratio is defined as [(DOPAC + HVA)/dopamine]. At day 5 of exercise, there was a significant effect of MPTP on dopamine and DOPAC levels and turnover ratio, compared with saline-treated animals (the asterisk represents significance at $p < 0.0001$). There was no significant effect of exercise on dopamine levels in either MPTP or saline-treated groups. Exercise at day 5 caused a significant decrease in turnover ratio and HVA levels in the MPTP plus exercise group compared with the MPTP plus no exercise group only (# represents significance at $p < 0.02$). At day 28 of exercise, there was a significant effect of MPTP on dopamine, DOPAC, and HVA levels, compared with saline-treated animals (the asterisk represents significance at $p < 0.0001$). There was a significant increase in straital dopamine levels in the saline plus exercise animals compared with the saline plus no exercise animals (+ represents significance at $p = 0.015$), but there was no significant effect of exercise on dopamine levels in the MPTP-treated groups.

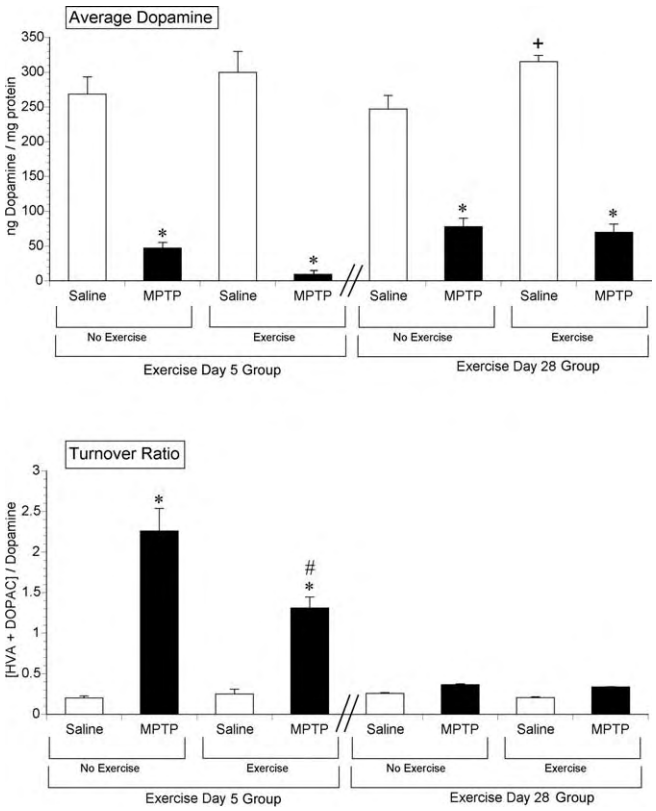


**Figure 3.** HPLC analysis of striatal dopamine levels and dopamine turnover. This figure shows data from Table 1 for striatal dopamine levels and turnover ratio at both 5 d of exercise (corresponding to 10 d postlesioning), and also at 28 d of exercise (corresponding to 42 d postlesioning). At day 5 of exercise, there was 82–98% depletion of dopamine in all MPTP-treated animals (exercise and no exercise) compared with their respective saline groups (asterisk represents significance at $p < 0.0001$). There were no significant differences in striatal dopamine levels between exercise and no exercise animals in either the MPTP- or saline-treated animals. Turnover ratio was significantly elevated in all MPTP- compared with saline-treated animals (asterisk represents significance at $p < 0.0001$). There was a significant decrease in turnover ratio in the MPTP plus exercise compared with the MPTP plus no exercise animals (hash mark represents significance at $p < 0.02$). At day 28 of exercise, there was >68–78% depletion of striatal dopamine levels in all MPTP-treated animals (exercise and no exercise) compared with their respective saline groups (asterisk represents significance at $p < 0.0001$). There was a significant increase in striatal dopamine levels in saline plus exercise compared with saline plus no exercise groups only (cross represents significance at $p = 0.015$). Exercise had no effect on turnover ratio. Error bars indicate SEM.

atum (there was an interaction of exercise with location). The mechanism of dopamine release induced by our intrastriatal stimulation was investigated by sequentially testing the evoked dopamine response in control aCSF (containing 2.4 mM $Ca^{2+}$ and 1.3 mM $Mg^{2+}$), reduced $Ca^{2+}$ aCSF (containing 0.5 mM $Ca^{2+}$ and 3.2 mM $Mg^{2+}$), and washout with normal aCSF followed by addition of aCSF containing 1 $\mu$M TTX. The reduced $Ca^{2+}$ aCSF decreased the evoked dopamine response to 25.85 ± 5.44% ($n = 3$) of control, which returned to the control amplitude with washout by normal aCSF. Subsequent addition of TTX completely blocked the evoked dopamine release ($n = 3$).

The decay rate of the dopamine signal evoked by intrastriatal stimulation was also compared for site 4 between each of the groups (see Materials and Methods for determining decay rate constant, $-k$). Site 4 was chosen, because this site showed the greatest exercise-induced difference for peak dopamine release in the MPTP-lesioned group. Exercise had an effect on the decay constant for the evoked dopamine signal ($-k$). *Post hoc* analysis revealed a significant slowing in dopamine decay rate in the MPTP plus exercise group ($n = 11$) compared with the MPTP alone group ($n = 7$; $p < 0.05$; $t$ test). A similar trend was seen between saline plus exercise group ($n = 11$) and the saline only group ($n = 11$; $p = 0.13$).

As shown previously, there was a significant reduction in striatal DAT and TH protein expression in MPTP plus exercise mice compared with MPTP mice ( $p < 0.05$) (Fisher et al., 2004). MPTP-treated mice were also shown to have a significant decrease in TH and DAT protein expression compared with saline-treated mice ( $p < 0.001$). No significant differences in TH and DAT protein were observed between saline plus exercise and saline mice. These data are shown in Figure 5. To determine whether changes in protein level were accompanied by changes in the relative expression of either TH or DAT mRNA transcript in midbrain dopaminergic neurons, *in situ* hybridization histochemistry in conjunction with grain counting of emulsion-dipped sections was used. Mice from all four groups were analyzed after the 28 d of exercise and these data are shown in Figure 6. MPTP lesioning caused a significant decrease in the relative expression of TH and DAT transcript expression in midbrain dopaminergic neurons ($F_{(3,12)} = 26.1$, $p < 0.001$ and $F_{(3,12)} = 37.29$, $p < 0.001$, respectively). Exercise caused a significant decrease in TH and DAT mRNA transcript expression in saline-treated but not in MPTP-treated mice. Specifically, although exercise had no overall effect on TH transcript expression ($F_{(3,12)} = 2.26$; $p = 0.133$), there was a significant interaction between exercise and MPTP lesioning ($F_{(3,12)} = 51.7$; $p < 0.001$). This interaction was because of a 28% reduction in TH mRNA expression in the saline plus exercise group compared with saline plus no exercise group. Exercise also caused a significant decrease in DAT transcript expression, ($F_{(3,12)} = 73.2$; $p < 0.001$), and there was a significant interaction between exercise and MPTP lesioning ($F_{(3,12)} = 33.7$; $p < 0.001$). This interaction was again because of a 43% reduction in DAT mRNA expression in the saline plus exercise group compared with the saline plus no exercise group.

As a measure of the integrity of the midbrain nigrostriatal dopaminergic neurons, we determined the number of TH-ir neu-

rons in the SNpc using unbiased stereological counting in mice from the saline, saline plus exercise, MPTP, and MPTP plus exercise groups ($n = 3$ per group) (Fig. 7). MPTP lesioning caused a significant decrease in the number of TH-ir SNpc neurons ($F_{(3,8)} = 106.9$; $p < 0.001$). However, exercise caused no significant effect on the number of TH-ir SNpc neurons ($F_{(3,8)} = 0.022$; $p = 0.885$), and there was no significant interaction between exercise and MPTP on the number of TH-positive neurons.

## Discussion

As reported previously, intensive treadmill exercise leads to improvement of motor performance in MPTP-lesioned (Tillerson et al., 2003; Fisher et al., 2004) and saline-treated mice (Fisher et al., 2004). In the present study, we also demonstrate that intensive treadmill exercise leads to increased latency to fall (improved balance) on the accelerating rotarod. This finding suggests that intensive treadmill exercise may, through adaptive changes of the basal ganglia and motor circuitry, lead to improvement in related motor tasks (balance) in both MPTP and saline-treated animals. Our findings indicate, however, that the beneficial effects of exercise are accompanied by differential effects on the dopaminergic system that may be dependent on the presence or absence of nigrostriatal injury (lesioned versus unlesioned). Specifically, although intense exercise improved motor performance in both groups, using HPLC analysis, we observed that this functional benefit was accompanied by a significant increase in total striatal dopamine after 28 d of exercise in saline plus exercise compared with saline plus no exercise mice only. There were no significant differences in total striatal dopamine levels in MPTP mice, examined after either 5 or 28 d of treadmill running, compared with their corresponding no exercise groups. In addition, exercise had no effect on the time course of dopamine return in MPTP-lesioned mice. Specifically, we observed a partial return of striatal dopamine in all MPTP-lesioned mice at day 28 compared with day 5, and this effect was observed to the same degree in exercise and no exercise mice, and in accordance with previous reports (Ricaurte et al., 1986; Bezard et al., 2000; Jakowec et al., 2004). Although HPLC analysis of total tissue catecholamines is an excellent measure of the total dopamine pool, including synaptic, extra-synaptic, vesicular, and cytoplasmic, it may not be an accurate estimate of the amount of dopamine released with activity (Garris et al., 1997; Yavich and MacDonald, 2000; Dentresangle et al., 2001).

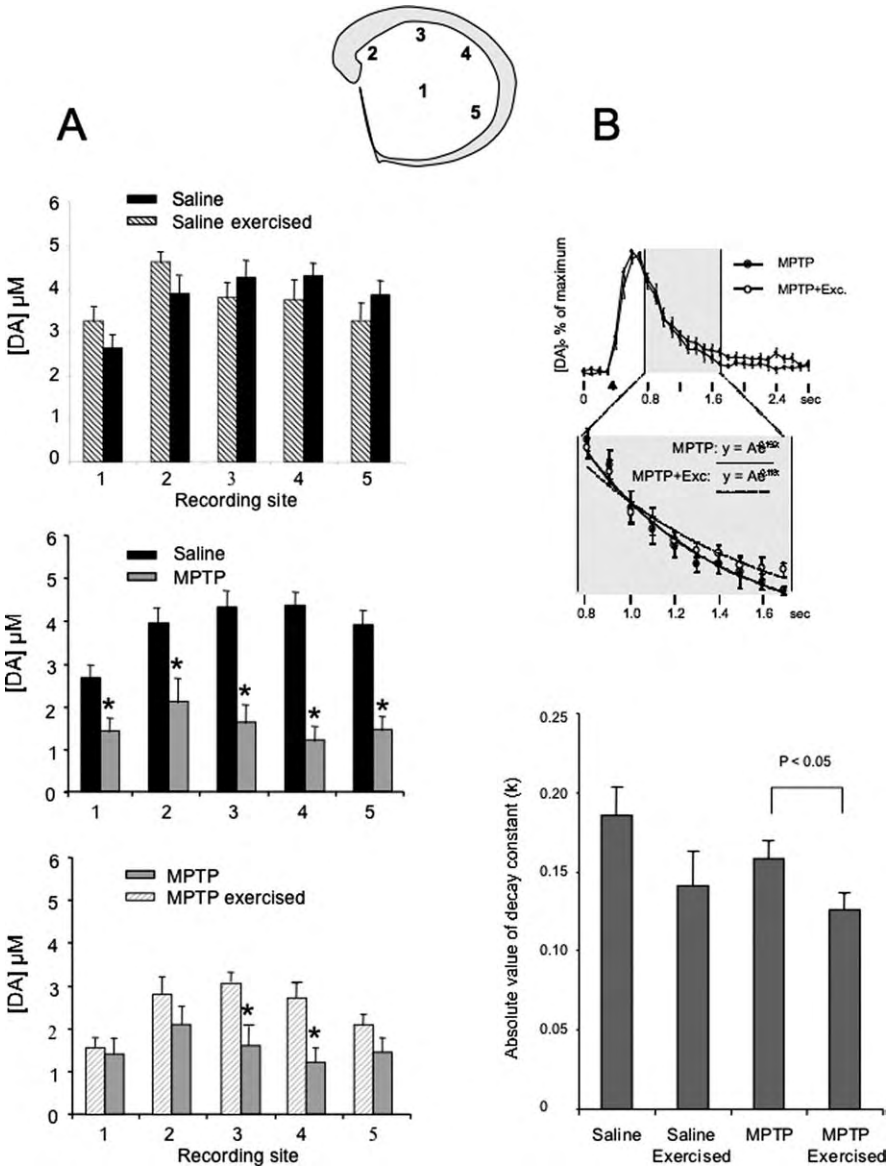


**Figure 4.** Analysis of dopamine release using fast-scan cyclic voltammetry. The amount of dopamine release was determined at five regions within the striatum (at bregma level ~1.00 to bregma 0.80) including (1) mid-striatum, (2) dorsomedial, (3) dorsal, (4) dorsolateral, and (5) ventrolateral in representative mice from all four groups (see brain slice insert). ***A***, Comparison of peak dopamine released by a single intrastriatal stimulus (200 A, 0.1 ms). Top, Data comparing saline and saline plus exercise mice. There was no significant difference in dopamine release in any region. Middle, Data comparing saline and MPTP groups. There was a significant decrease in dopamine release in MPTP mice and the asterisk represents significant difference compared with saline ( $p < 0.001$). Bottom, Data comparing MPTP and MPTP plus exercise mice. There was a significant increase in dopamine release in the MPTP plus exercise mice at dorsal sites 3 and 4 compared with MPTP mice, and the asterisk represents significant difference ( $p < 0.05$). ***B***, Comparison of dopamine signal (peak and decay) evoked by intrastriatal stimulation. Top, A plot of average time to peak and decay in the dopamine signal at electrode position 4 for MPTP plus no exercise ($n = 7$, filled circles) and MPTP plus exercise ($n = 11$, open circles). The intrastriatal stimulus was delivered at the time indicated by the filled triangle (between 0 and 0.8 s). Data points are normalized to the peak-evoked dopamine signal. The middle panel illustrates the decay phase of the graph (shaded area), which was fit with a single exponential function. Best fit for the average data from MPTP plus no exercise mice (solid line) and MPTP plus exercise mice are shown (dotted line). The bottom panel illustrates the averages of the decay constant ($k$) obtained by the exponential fit of the decay phase for each recording and are shown for each group (mean ± SEM). MPTP plus exercise mice had a significantly lower decay constant compared with MPTP plus no exercise mice ( $p < 0.05$; $t$ test). A similar trend was seen in the saline plus exercise compared with the saline plus no exercise animals ( $p = 0.13$; $t$ test). Exc, Exercise.

In contrast to total tissue HPLC analysis, using fast-scan cyclic voltammetry in striatal slices of mice exercised for 28 d, we observed an exercise-dependent increase in stimulus-evoked dopamine release in MPTP plus exercise compared with MPTP plus

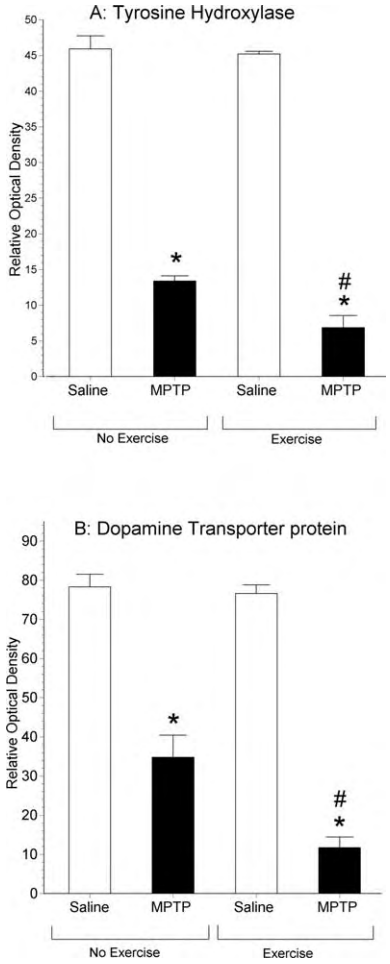


**Figure 5.** Analysis of striatal TH and DAT protein. ***A***, ***B***, The relative expression of striatal TH (***A***) and DAT (***B***) was determined using immunohistochemistry and computer-assisted image analysis after 28 d of exercise. Sections at the level of the midstriatum (≥6–8 sections from 4 different mice within each group) were immunostained with an antibody against TH or DAT. Sections were scanned and the relative optical density mean plus SEM for each group was determined. MPTP causes a significant decline in the level of expression of striatal TH and DAT protein compared with saline-treated mice, and the asterisk represents significance at $p < 0.001$. There is a significant interaction between exercise and MPTP, with a significant decrease in the MPTP plus exercise compared with the MPTP plus no exercise mice, and the hash mark represents significance at $p < 0.05$.

no exercise mice. Our results show that the stimulus-evoked dopamine release is vesicular, because it was decreased by reduced extracellular $Ca^{2+}$ and it was completely blocked by blockage of $Na^{+}$ channels by TTX as reported previously (Cragg and Greenfield, 1997; Chen and Rice, 2001). This exercise-dependent effect was not seen in the saline plus exercise compared with saline plus no exercise mice. Interestingly, the exercise effect on stimulus-evoked dopamine release in the MPTP plus exercise mouse was most pronounced within the dorsolateral striatum. A possible explanation for this exercise and regional specific effect may be because of use-dependent forms of synaptic plasticity. The high degree of engagement required from the dorsolateral striatum for forelimb and hindlimb movement during treadmill exercise is supported by studies demonstrating a selective increase in blood flow and metabolic activity within the dorsolateral striatum during treadmill exercise (Cospito and Kultas-Ilinsky, 1981; Ebrahimi et al., 1992; Brown and Sharp, 1995; Holschneider et al., 2003; Nguyen et al., 2004).

Fast-scan cyclic voltammetry is a technique that allows for precise anatomical targeting and examination of evoked dopamine release at a number of distinct sites within the same brain slice during a single recording session, thus providing the ability to detect regional differences in dopamine release. Although an advantage of *in vivo* recording is the retention of intact afferent pathways, stimulation of the medial forebrain bundle (MFB) may be variable across these afferent fibers and lead to differences in dopamine release even within a hundred micrometers between striatal recording sites (Day et al., 1989). Brain slices may not retain an intact nigrostriatal pathway but it does avoid variation in evoked release because the entire recording field is excited and provides a reasonable tool to investigate the way this neurotransmitter system responds (Patel and Rice, 2006). In a set of control experiments, we verified that the release of dopamine was in fact mediated through an action potential by carrying out the fast-scan cyclic voltammetry studies in the presence of the sodium channel blocker TTX.

In agreement with our study, compensatory changes in dopamine release have been reported after injury of the dopaminergic system using 6-OHDA lesioning and in patients with PD during motor exercise (Zhang et al., 1988; Garris et al., 1997; Ouchi et al., 2001). In addition, a recent study from O'Dell et al. (2006) using 6-OHDA striatal lesioning in the rat, supports our findings that improvement in motor performance may not necessarily be accompanied by changes in total striatal dopamine levels after exercise. Specifically, this group reported exercise-enhanced return of motor behavior without the return of striatal dopamine levels to the prelesioning baseline (O'Dell et al., 2006). Our studies and those reported by O'Dell et al. (2006) are in contrast to others that show partial return of striatal dopamine in neurotoxicant-lesioned rodent models undergoing exercise (Tillerson et al., 2003). One possible explanation for this discrepancy may be because of differences in the MPTP-lesioning regimen and exercise paradigm. In our study, we used an MPTP-lesioning schedule that resulted in a moderate degree of nigrostriatal dopaminergic cell loss and initiated exercise 5 d after MPTP lesioning, when cell death is complete. Conversely, studies demonstrating a partial dopamine return have used milder lesioning regimens and have initiated treadmill running immediately (within 24 h) after lesioning, when the potential for exercise-induced downregulation of the DAT may influence both the amount of neurotoxin uptake and the degree of lesioning (Gainetdinov et al., 1997). Unbiased stereological counting of midbrain dopaminergic neurons in our study indicates that exercise-induced downregulation of DAT appears independent of the number of surviving substantia nigral neurons.

Using immunohistochemical analysis, we observed that 28 d of intense exercise leads to the downregulation of both DAT (a protein responsible for uptake and clearance of dopamine from the extracellular space) and TH (an enzyme responsible for the rate-limiting step in dopamine biosynthesis) (Jackson-Lewis et al., 1995; Fisher et al., 2004) protein. This finding is supported by our previous work demonstrating suppression of both TH and DAT protein in MPTP plus exercise mice using both Western immunoblotting and immunohistochemical staining, two complementary techniques to examine protein expression levels and anatomical patterns of distribution (Burke et al., 1990; Jakowec et al., 2004). To verify that this decrease of striatal DAT and TH protein was not because of exercise-induced nigrostriatal cell death, the number of midbrain dopaminergic neurons based on TH immunoreactivity were determined using unbiased stereological counting techniques. Comparison of the exercise and no exercise groups showed no significant differences in the total

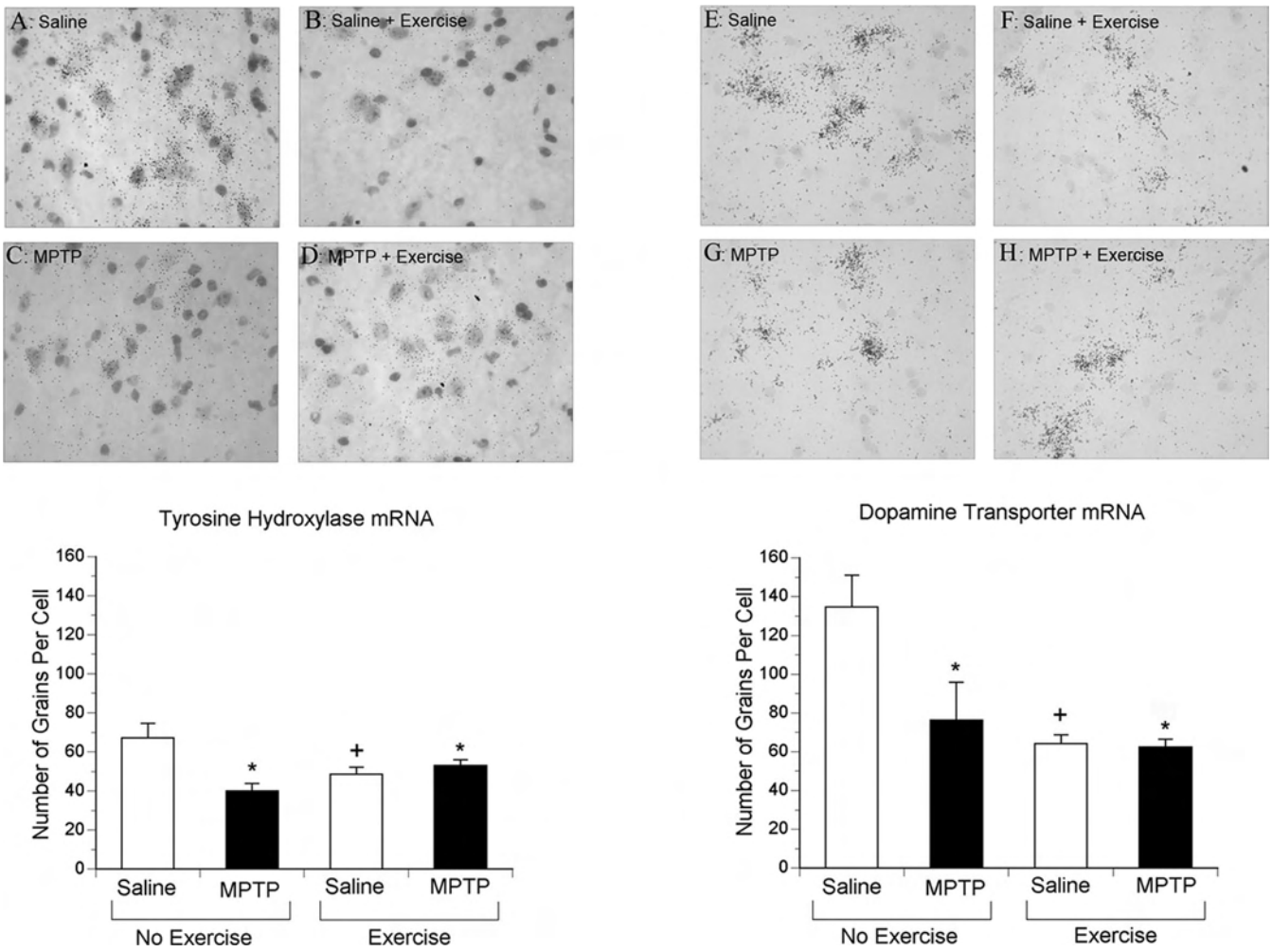


**Figure 6.** Analysis of TH and DAT mRNA in midbrain nigrostriatal neurons. The relative expression of TH and DAT mRNA in midbrain dopaminergic neurons were determined using *in situ* hybridization histochemistry followed by grain counting of emulsion-dipped sections. Representative sections showing neurons for the determination of TH mRNA expression are shown in panels ***A*** (saline), ***B*** (saline plus exercise), ***C*** (MPTP), and ***D*** (MPTP plus exercise). The respective analysis of the data for either TH or DAT is shown in the graphs below each set of images. At least 120 neurons with grains were counted for each treatment group. MPTP lesioning caused a significant decrease in both TH and DAT transcript compared with the saline-treated groups, and the asterisk represents significance at $p < 0.001$. Statistical differences were seen between the saline and saline plus exercise groups for both the TH and DAT transcript, and the cross represents significance at $p < 0.001$. There were no significant differences in transcript expression between the MPTP and MPTP plus exercise groups. Representative sections showing neurons for determination of DAT mRNA expression are shown in ***E*** (saline), ***F*** (saline plus exercise), ***G*** (MPTP), and ***H*** (MPTP plus exercise). Error bars indicate SEM.

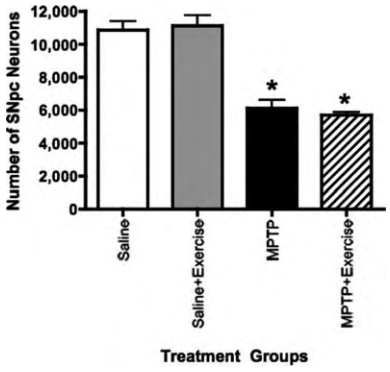


**Figure 7.** Determination of SNpc dopaminergic cell number. Unbiased stereological counting of TH-ir neurons was performed in mice ($n = 3$ per group) from all four groups at the end of the 28 d running regimen. MPTP lesioning resulted in an ~50% decline in SNpc dopaminergic neurons. There was no statistically significant difference between the saline and the saline plus exercise mice as well as no difference between the MPTP and MPTP plus exercise groups, indicating that there was no effect of exercise on the number of SNpc dopaminergic neurons. The asterisk represents significant difference from the saline group ( $p < 0.0001$).

number of midbrain dopaminergic neurons between either MPTP and MPTP plus exercise or saline and saline plus exercise mice, which indicated no additional cell death induced by exercise. This finding suggests that both DAT and TH proteins are downregulated by exercise in surviving midbrain dopaminergic neurons after MPTP lesioning. Downregulation of DAT may lead to increased synaptic availability and represent a compensatory effect of the dopaminergic system because of exercise in the injured state. Indeed, using fast-scan cyclic voltammetry analysis of the decay rate of the dopamine signal evoked by a single stimulus in striatal brain slices, we observed a slower dopamine decay rate in the MPTP plus exercise compared with the MPTP plus no exercise mice, possibly related to the decline in DAT. The increase in dopamine release seen in exercised mice could also reflect more diffusion of dopamine from synapses distant from the recording site when DAT is reduced (Cragg and Rice, 2004). The exercise-induced increase in evoked dopamine release in the dorsolateral striatum together with a DAT-related lengthening of the dopamine signal may underlie one mechanism by which intense exercise facilitates normal motor circuitry because corticostriatal

synaptic plasticity, both LTD and LTP, is modulated by dopamine. In the dorsolateral striatum, the increased release and decreased decay of dopamine could play an important role in maintaining normal LTD (Calabresi et al., 1992; Smith et al., 2001; Akopian and Walsh, 2007).

The expression of DAT and TH proteins, as well as the level of striatal dopamine, is closely linked. For example, DAT knock-out mice have been shown to express reduced striatal TH protein (Jaber et al., 1999). Although the precise link between DAT and TH is unclear, it is speculated that the increased synaptic bioavailability of dopamine, through the downregulation of DAT, may lead to increased activation of the dopamine autoreceptor, leading to the downregulation of TH (Fauchey et al., 2000). In support of our findings, studies with neuroimaging have shown rapid downregulation of DAT in patients with PD undergoing motor activity (walking) (Ouchi et al., 2001). A similar downregulation of DAT with activity can be induced in young mice exposed to environmental enrichment and this can in fact prove neuroprotective against MPTP lesioning (Bezard et al., 2003).

In our study, we used *in situ* hybridization histochemistry to examine the level of expression of mRNA transcripts of TH and DAT in surviving nigrostriatal dopaminergic neurons to determine whether alterations in transcription could account for exercise-induced suppression of striatal protein. We found a reduction in expression of TH and DAT mRNA in saline plus exercise compared with saline mice but not in MPTP plus exercise compared with MPTP mice. The effect of exercise on mRNA transcript expression may be dependent on the presence of a previous injury to the nigrostriatal dopaminergic neurons. MPTP can lead to reduction in TH mRNA expression in surviving midbrain neurons, and this may mask potential effects of exercise (Jakowec et al. 2004). Because exercise-induced changes in striatal protein cannot be accounted for by changes in transcript expression in surviving nigrostriatal dopaminergic neurons in MPTP mice, exercise may act through alternative mechanisms that influence TH and DAT rates of translation, axonal translocation, or turnover.

In conclusion, our results indicate that intensive treadmill exercise leads to improvement of motor performance in both MPTP and saline mice and this behavioral improvement is also observed in a related motor task. The beneficial effects of exercise may be because of alterations in dopaminergic neurotransmission, which may be different between the normal and injured basal ganglia. Exercise leads to compensatory changes in the MPTP-lesioned mouse resulting in increased synaptic dopamine availability through increased release, reduced uptake, and decrease in decay. In saline mice, exercise effects may be through elevated dopamine levels because of increased biosynthesis through increased TH activity. Currently, the molecular mechanism linking exercise and dopaminergic neurotransmission are unknown. However, reports in the literature suggest a role for neurotrophic factors, such as brain-derived neurotrophic factor, fibroblast growth factor, or glial-derived neurotrophic factor, which through activation of down-stream pathways, such as protein kinases, may influence synaptic plasticity and terminal neurotransmission (Gomez-Pinilla et al., 1997, 2002; Cohen et al., 2003). The glutamatergic corticostriatal pathway may be another candidate system involved in exercise-related alterations in dopamine release, because it is known to be an important modulator of dopamine release in the striatum, and our previous work has shown alterations in the density of striatal glutamate immunolabeling in the MPTP plus exercise mice compared with sedentary MPTP-lesioned mice (Molteni et al., 2002; Fisher et al., 2004; Dietrich et al., 2005). Overall, these results indicate that the recovery of motor behavior can in fact occur through novel compensatory mechanisms within the basal ganglia and that the mechanisms of this recovery may be different in the lesioned compared with the nonlesioned basal ganglia. These findings may be important in treating neurodegenerative disorders of the basal ganglia, including Parkinson's disease, in which the enhancement of neuroplasticity through exercise may lead to altered dopaminergic availability (release and uptake), which in turn may play a more critical role in maintaining normal synaptic connections than the restoration of absolute dopamine levels.

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Final

# Nonhuman Primate Models of Parkinson's Disease and Experimental Therapeutics.

Giselle M. Petzinger, MD, Daniel M. Togasaki, MD/PhD, Garnik Akopian, MD, John P. Walsh, PhD, and Michael W. Jakowec, PhD.

Department of Neurology, and the Andrus Gerontology Center,
University of Southern California, Los Angeles, California.

## Introduction

The nonhuman primate serves as an important model for understanding the pathophysiology of the basal ganglia, evaluating new treatment modalities for neurodegenerative disorders affecting this region, especially Parkinson's disease (PD), and may provide a key component in finding the cure for PD. The nonhuman primate model generated through either neurotoxicant or surgical lesioning has been most commonly used for experimental therapeutic studies in PD, in particular for identifying new symptomatic strategies primarily targeting the dopaminergic system, as well as those neurotransmitter systems known to modulate dopamine (including serotonin, glutamate, acetylcholine, and noradrenalin). The model has also been extremely valuable in providing important insights into the pathophysiology and treatment of levodopa-induced dyskinesia, a disabling complication of long-term levodopa use in PD. In addition, the model has provided fertile ground for investigating innovative therapeutic approaches, such as gene therapy using vector delivery systems, tissue transplantation, neurotrophic factor delivery, and deep brain stimulation. Most important, the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned nonhuman primate model has been valuable for understanding basal ganglia function in both the normal and injured state by providing insights into the role of dopamine in motor learning, motor control, and synaptic function that will guide the development of new therapeutic approaches for PD and related disorders. The strength of the nonhuman primate as a model of PD lies in its similarities to the human condition for; (i) clinical phenomenology; (ii) response to dopamine replacement therapy; and (iii) neuroanatomy. These strengths have been confirmed by 25 years of investigations that have yielded valuable insights toward the treatment of PD and normal basal ganglia function.

Although the nonhuman primate model has been important in identifying many new treatments for PD, a number of novel preclinical studies, including pharmacological targeting of non-dopaminergic neurotransmitter systems, vector-based gene therapy, and tissue transplant approaches did not accurately predict efficacy in clinical trials. There are many factors that may contribute to this lack of a translational success including; (i) limitations in our knowledge regarding basal ganglia function in both the normal and diseased state; (ii) limited understanding of the pharmacokinetics and bioavailability of novel compounds; (iii) poorly elucidated adverse effects, including cardiovascular and cognitive changes; (iv) limited understanding of molecular mechanisms of the treatment intervention; and (v) differences between preclinical and clinical study design. Still, studies have shown that the nonhuman primate model addresses many important questions in addition to efficacy, including safety and tolerability, and technical issues addressing feasibility. More important, the model can be used to understand the underlying molecular mechanisms responsible for the success or failure of any new therapy, and consequently becomes a valuable conduit in bi-directional translational medicine from bench to bedside and bedside to bench. The value of information gained from studies using the nonhuman primate model of PD resides in understanding the details and differences of any one of the several distinct nonhuman primate models that may be used in the experimental paradigm. Important parameters that may vary amongst the many different nonhuman primate models include the lesioning regimen, behavioral assessments, and the pathological, neurochemical and molecular features of the model, some of which may be dynamic and display altered plasticity weeks to months post-lesioning.

The primary goal of this chapter is to review the more commonly used nonhuman primate models for PD, including; (i) behavioral assessments used to measure parkinsonian motor features or abnormal involuntary movements that are attributed to levodopa; (ii) the different types and subtypes of neurotoxicant models; (iii) the utility of the model in pharmacological and non-pharmacological experimental therapeutic studies; and (iv) examples of how studying the models themselves helps to understand basal ganglia function and physiology. While the vast majority of nonhuman primate models of PD utilize the neurotoxicant MPTP, which selectively destroys nigrostriatal dopaminergic neurons, other neurotoxicants with selectivity for midbrain dopaminergic neurons, such as 6-hydroxydopamine, methamphetamine, and proteasome inhibitors have been utilized. The rationale in developing these models is primarily based on success in rodents where much of the basic foundations have been established. In understanding the details of each model, the investigator can appreciate more fully the individual strengths and limitations, design studies that are tailored to the model,

effectively interpret outcome measures and compare them to findings from other models, and guide the design, expectant outcome measure, and interpretation of potential clinical studies.

**Assessment of Motor Behavior in Nonhuman Primate Models of PD**

New pharmacological and non-pharmacological agents evaluated as possible treatments in nonhuman primate models of PD require the observation and quantification of motor behavior(s) associated with the parkinsonian state. These models display significant bradykinesia as the primary clinical feature. They may also demonstrate balance problems, ataxia, tremor, freezing, and changes in posture. Several approaches have been used to quantify motor activity, including: (i) clinical rating scales; (ii) cages designed to measure animal movement (typically by having the animal break infrared beams traversing the cage); and (iii) personal activity monitors affixed to the animals (often based upon a small accelerometer). Of these methods, clinical ratings are most able to specifically target different types of clinical phenomenology, thus ensuring that the behavior of interest is the one being assessed. These ratings, however, are only semi-quantitative, and have a strong subjective component. Furthermore, conducting the ratings is labor intensive. In contrast, the more objective methods using mechanical and electronic technology provide quantitative data that can undergo parametric statistical analyses, but at the expense of losing the ability to target different subtypes of movement, such as locomotion or choreoathetosis. More recently, video-based systems with computerized analysis have become available; these systems can provide sophisticated quantitative analyses while simultaneously distinguishing types of movements, but they tend to require significant financial investment.

A primary concern is that the method of data collection is valid, reliable, and measures the motor behavior of interest. Few methods have been shown to meet these criteria. Most of the automated techniques measure locomotor activity (e.g., infrared beam breaks within the cage) or general motor activity, which also includes a non-locomotor component. It is important to keep in mind that different types of motor activity might reflect different clinical phenomena. For example, a decrease in locomotion is generally accepted as being an accurate reflection of parkinsonian bradykinesia. In contrast, a change in non-locomotor activity might correlate, for example, with changes in levels of levodopa-induced dyskinesias, a very different entity.

Another consideration in choosing a behavioral monitoring method is its ability to be applied repeatedly in a serial fashion to generate a time course. Because behavior is highly variable, the additional data points gathered in constructing a time course allows for much greater power in the statistical analyses. Furthermore, it is often difficult to know *a priori* how long after drug administration the peak effect will occur: making a series of repeated measurements throughout the entire duration of the behavioral change provides a more accurate assessment than making one measurement at a single time point when the peak effect is *presumed* to occur. This assumes even greater importance in studies using multiple drugs, leading to complex pharmacokinetic interactions. For example, using one drug to suppress levodopa-induced dyskinesias could lead to uncertainty about what aspect of the behavior might be affected: the duration of the dyskinesias might be shortened, the severity of the peak effect might be lessened, or both might occur. Automated methods have an obvious advantage for making repeated measurements. Clinical ratings can be assessed from video recordings, but attempting to perform serial ratings live at cage-side is decidedly more difficult.

**Clinical Rating Scales**

A number of investigators have developed clinical rating scales for the different models. It is essential that these scales must be tested for inter-rater reliability, be validated against other behavioral measures, and be sensitive enough to capture response to intervention. Most rating scales have been based upon the human clinical signs assessed in the Unified Parkinson's Disease Rating Scale (UPDRS), with minor modifications made to suit the primate species. A review of rating scales for parkinsonism in nonhuman primates is available (Imbert *et al.*, 2000). For example, we have developed a clinical rating scale specific for motor behaviors that are affected by MPTP-lesioning and are responsive to levodopa replacement therapy (Petzinger *et al.*, 2001). Rating scales have also been devised to evaluate the presence and intensity of motor complications specifically for the squirrel monkey that takes into consideration the evaluation of, specifically levodopa-induced dyskinesia (Petzinger *et al.*, 2001; Tan *et al.*, 2002) and reviewed by Bezard and colleagues (Imbert et al., 2000).

The main disadvantages of clinical rating scales are that their application is much more labor-intensive than automated methods, and that assigning ratings is a subjective process. The main advantage is that the rating scales can be designed to target features of the clinical condition that resemble human parkinsonism, so that the relationship to PD is straightforward, readily apparent to clinical neurologists, and thus less controversial. One precaution to keep in mind, however, is that the manifestations of parkinsonism in the nonhuman primate might not match those seen in patients with PD. For example, rest tremor does not appear to occur in most nonhuman primate species, so that attempts to include ratings for this would be futile. On the other hand, action tremor, which is not felt to be a sign of parkinsonism in humans, appears with great consistency in MPTP-lesioned squirrel monkeys, so omitting an assessment of this behavior might ignore an important component of the syndrome in that species. This controversy is an area of debate. In any case, the development of a clinical rating scale should be grounded in comprehensive observation of the full behavioral repertoire of the animal in that model.

**Automated Behavioral Observation Methods**

Another approach to measuring parkinsonian motor behavior is through the application of activity monitors either applied to the home cage or attached to the primate itself. Cage-based activity monitors are most commonly grounded in technologies similar to those used in rodent studies where movements are counted when a subject breaks infrared beams traversing the cage. These techniques distinguish between repetitive breaking of a single beam and consecutive breaks of different beams, thus allowing the apparatus to distinguish translational movement of the animal through space, which provides a measure generally accepted to represent locomotor activity. A recent technique utilizes telemetric methods to detect a probe implanted in the subcutaneous tissue of the animal to monitor locomotion. Personal activity monitors, such as the small accelerometers manufactured by Minimitter Company (Bend, OR), can be attached to the subject either using a collar, jacket, or straps. Most nonhuman primates require training or habituation to adapt to these encumbrances. These personal monitors measure general motor activity, so that the output encompasses both translational movement through space and movements performed with the animal stationary in the cage. Distinguishing between the different movement types, however, is impossible from the raw data from this device alone.

Newer types of activity measures have been derived from the advances in computer-based video technologies. These methods span a wide spectrum. At the low end are ones requiring minimal expense but provide little or no technical support and yield relatively crude measures. In contrast, there are other, commercially produced systems that can track each of the animals' limbs separately, recording position and velocity, and then provide sophisticated analyses of specific movement subtypes, with the trade-off being that acquiring these systems requires substantial financial investment.

Each of these systems has advantages and disadvantages. Choosing which method to use for measuring activity depends upon the specific requirements of the study design. In many cases, complementary methods are combined. For example, an automated system is often used in conjunction with a clinical rating scale. We suspect that, in the future, a popular approach will be simultaneous application of a video-based computerized method with ratings performed on the video images.

**Data Analysis**

Behavioral experiments tend to produce large amounts of data, so that the statistical analysis of the results can be a daunting task. In general, these analyses are performed using statistical software packages, such as SPSS (SPSS Inc., Chicago, IL) or SAS/STAT (SAS America Inc., Cary, NC). The statistical analysis of time using sophisticated courses required application of repeated measures analysis of variance. This proved relatively straightforward for parametric variables, but was trickier for nonparametric variables, such as clinical ratings. For an example of one way to handle such analyses, see (Togasaki *et al.*, 2005).

For experiments examining the suppression of levodopa-induced dyskinesias, the analysis can be complex. For example, studies require that drugs used to treat dyskinesia both decrease dyskinesia and do so without causing a corresponding exacerbation of parkinsonism. This can be examined by performing correlation analyses in which the improvement in levodopa-induced dyskinesias is correlated with the change

in the severity of parkinsonism; this can determine whether there is a selective effect upon dyskinesias (Hsu *et al.*, 2004).

**Non-motor Features in MPTP-Lesioned Nonhuman Primates**

A potentially valuable application of the MPTP-lesioned nonhuman primate is in elucidating the anatomical sites and neurotransmitter systems involved in non-motor behavior. Although the substantia nigra pars compacta (SNpc) is certainly the most vulnerable region affected by MPTP, there are varying degrees of injury and cell death in other brain areas including the ventral tegmental area (VTA), retrorubal field, nucleus Basalis of Meynert, raphe nucleus, and locus ceruleus (Forno *et al.*, 1986a; Forno *et al.*, 1986b; Mitchell *et al.*, 1985). The fact that these anatomical regions mediate non-motor behavior and that MPTP can alter their neurotransmitter systems (including serotoninergic, cholinergic, and noradrenergic) suggests that non-motor features may be observed in these models (Friedman and Mytilineou, 1990; Namura *et al.*, 1987; Perez-Otano *et al.*, 1991). Unfortunately, there have been a limited number of studies in the MPTP-lesioned nonhuman primate examining non-motor features, which include cognitive impairment (executive function), sleep disorders, and affective behaviors such as depression and anxiety (Decamp and Schneider, 2004; Schneider *et al.*, 1995). For example, cognitive changes, including reduced executive function and attention, are evident with early parkinsonian features after chronic low dose MPTP administration, which might be associated with injury of dopaminergic pathways to the prefrontal cortex (Decamp and Schneider, 2004; Schneider, 1990ab; Schneider and Pope-Coleman, 1995; Slovin *et al.*, 1999b). In addition, other cognitive deficits in working memory, which might reflect dopaminergic injury to the hippocampus, can be observed (Decamp *et al.*, 2004). Alterations in sleep organization patterns have also been observed in MPTP-lesioned monkeys, with more severe disturbances in animals receiving greater amount of MPTP (Almirall *et al.*, 1999).

**The MPTP-lesioned Nonhuman Primate Model**

The most common model of PD in the nonhuman primate is formed by the administration of the neurotoxicant MPTP. The major strengths of this model include: (i) parkinsonian features that closely resemble the human condition; (ii) the robust response to dopaminergic therapy; (iii) manifestation of levodopa-induced motor complications including dyskinesia and wearing-off; and (iv) neuroanatomy and physiology features that are similar to that in humans. This model has been well validated over the last 25 years as reflected in its ability to successfully predict the efficacy of drug treatments in patients with PD. In the following sections we present the history of MPTP, features of the MPTP nonhuman primate model, the different subtypes of models generated using various lesioning regimens, and finally, examples of the utility of the model for evaluating new therapeutic agents and strategies, and for providing insights into basal ganglia function in the normal and parkinsonian states.

**A Brief History of MPTP**

The inadvertent self-administration of MPTP by heroin addicts in the late 1970s and early 1980s induced an acute form of parkinsonism whose clinical features were indistinguishable from idiopathic PD (Davis *et al.*, 1979; Langston *et al.*, 1983). The initial cohort of 7 parkinsonian symptomatic patients, from a population of over 400 heroin addicts exposed to MPTP, displayed severe bradykinesia, tremor, and impaired balance. As in idiopathic PD, these patients demonstrated an excellent response to levodopa and dopamine agonist replacement therapy. Positron emission tomography (PET) imaging using $^{18}$F-DOPA showed significant reduction in uptake similar to late-stage PD (Calne *et al.*, 1985; Snow *et al.*, 2000; Vingerhoets *et al.*, 1994). The patients also developed levodopa-related motor complications (including dyskinesia and wearing-off). Interestingly, motor complications developed within weeks of starting dopamine replacement therapy, compared to years for idiopathic PD (Ballard *et al*., 1985). The rapidity with which these motor complications appeared was thought to reflect the severity of SNpc neuronal degeneration induced by the MPTP exposure. Although tragic, this remarkable serendipitous occurrence provided a major breakthrough in neurological disorders research with the identification of a model of disease fulfilling Koch's postulate in the human condition.

Immediately following its identification, MPTP was administered to both rodents and nonhuman primates and some of the most valuable animal models of PD were established. MPTP-lesioned nonhuman primate models encompass a wide spectrum of species including the squirrel monkey (*Saimiri sciureus*) (Langston *et al.*, 1984), long-tailed macaque or cynomologus (*Macaca fascicularis*) (Mitchell *et al.*, 1985), rhesus macaque (*Macaca mulatta*) (Burns *et al.*, 1983; Chiueh *et al.*, 1984b; Markey *et al.*, 1984), Japanese macaque (*Macaca fuscata*) (Crossman *et al.*, 1985; Jenner *et al.*, 1986), bonnet monkey (*Macaca radiata*) (Freed *et al.*, 1988), owl monkey (*Aotus trivirgatus*) (Collins and Neafsey, 1985), baboon (*Papio papio*) (Hantraye *et al.*, 1993; Moerlein *et al.*, 1986), African green monkey or vervet (*Chlorocebus aethiops* formerly *Ceropithecus aethiops*) (Taylor *et al.*, 1994), pig-tail macaque (*Macaca nemestrina*) (Chefer *et al.*, 2007), and common marmoset (*Callithrix jacchus*) (Jenner et al., 1986). The administration of MPTP to the nonhuman primate results in parkinsonian symptoms including bradykinesia, postural instability, freezing, stooped posture, and rigidity. Although postural and action tremors have been observed in many species after MPTP treatment, a resting tremor, characteristic of PD, is less commonly documented (Hantraye et al., 1993; Raz *et al.*, 2000; Tetrud *et al.*, 1986).

The mechanism of MPTP toxicity has been thoroughly investigated. The meperidine analog MPTP is converted to 1-methyl-4-pyridinium ($MPP^+$) by monoamine oxidase B. $MPP^+$ acts as a substrate of the dopamine transporter (DAT) and is selectively taken up by the dopaminergic cells of the SNpc, leading to the inhibition of mitochondrial complex I, the depletion of ATP, and cell death. In mice and nonhuman primates MPTP selectively destroys dopaminergic neurons of the SNpc, the same neurons affected in PD (Forno *et al.*, 1993; Jackson-Lewis *et al.*, 1995; Langston et al., 1984). Similar to PD other catecholaminergic neurons, such as those in the VTA and locus coeruleus, may be affected albeit to a lesser degree (Forno, 1996; Forno et al., 1986b; Mitchell et al., 1985). In addition, dopamine depletion occurs in both the putamen and caudate nucleus. Whether the putamen or caudate nucleus is preferentially lesioned may depend on animal species and regimen of MPTP administration (Bezard *et al.*, 2000; Kalivas *et al.*, 1989; Ricaurte *et al.*, 1986).

Unlike PD, Lewy Bodies have not been consistently documented. Animal age and route of MPTP administration are two factors that may influence the development of Lewy Bodies. Specifically, eosinophilic inclusions (resembling Lewy Bodies) have been described in aged MPTP-lesioned squirrel and cynomologus monkeys (Forno et al., 1986b; Kiatipattanasakul *et al.*, 2000). In humans, autosomal recessive juvenile parkinsonism (AR-JP) due to mutations in the *parkin* gene do not develop Lewy Bodies (Shimura *et al.*, 2000) whereas Lewy Bodies are common (and serve as the pathological hallmark) in idiopathic PD in the aged brain (Jellinger, 2001). The time course of MPTP-induced neurodegeneration is rapid, and therefore, represents a major difference from idiopathic PD, which is a chronic progressive disease manifesting over several years. Modifications in the MPTP-lesioning regimen, such as chronic treatment in the presence of probenecid or the delivery of alpha-synuclein via stereotactic targeting of expression vectors have been reported to promote the formation of intracellular protein aggregates reminiscent of Lewy Bodies (Eslamboli *et al.*, 2007; Meredith *et al.*, 2002). The requirement of Lewy Body formation in animal models is an issue of debate since they might be neurotoxic or they might be neuroprotective (Bodner *et al.*, 2006; Calne and Mizuno, 2004).

Following the administration of MPTP, the non-human primate progresses through acute (hours), sub-acute (days) and chronic (weeks) behavioral phases of toxicity that are due to the peripheral and central effects of MPTP. The acute phase occurs within minutes after MPTP administration and is characterized by sedation, and a hyper-adrenergic state. This state may also include hyper-salivation, emesis, exaggerated startle, seizure-like activity, and dystonic posturing of trunk and limbs (German *et al.*, 1988; Irwin *et al.*, 1990; Jenner and Marsden, 1988; Jenner et al., 1986; Petzinger and Langston, 1998). The sub-acute phase generally occurs within hours and persists for several days and may be due to the peripheral actions of MPTP on the autonomic nervous system and peripheral organs such as the liver, kidney, and heart (see below) (Petzinger and Langston, 1998). Weight loss, altered blood pressure, and hypothermia may occur, requiring oralgastoral tube feeding and placement in an incubator to stabilize body temperature. In addition, elevated liver transaminases and creatinine phosphokinase may develop; reflecting impaired liver function and muscle breakdown. Behaviorally, these animals may appear prostrate and cognitively impaired. Occasionally, animals may demonstrate self-injurious behavior such as finger biting and hyper-flexion of the neck and trunk with head banging. Assessment of parkinsonian features may be confounded by alterations in the general health of the animal. The chronic phase starts within days to weeks after MPTP administration. It is

characterized by the stabilization of body weight and temperature as well as the normalization of blood chemistries such as hepatic enzymes. Parkinsonian features clearly emerge and remain stable for weeks to months or longer. Similar to PD, the MPTP-lesioned nonhuman primate responds to traditional anti-parkinsonian therapies such as levodopa and dopamine receptor agonists; in some cases severely lesioned animals may require such an intervention to sustain survival. The degree of behavioral stability may be predicted in part by the initial degree of behavioral impairment as observed between the sub-acute to chronic phases. Animals with greater behavioral impairments require a longer period of time for recovery. Behavioral improvement after MPTP administration has been reported in most species of nonhuman primates (see below). In general the behavioral response to MPTP-lesioning may vary both within and between species. One must be cautious in simply extrapolating findings from one species or strain to another. For example, many, but not all, strains of mice are sensitive to the effects of MPTP, whereas rats are almost completely resistant (Riachi *et al.*, 1988; Zuddas *et al.*, 1994). This variability due to age and species phylogeny also applies to the nonhuman primate. For example, Old World monkeys (such as rhesus, *Macaca mulatta* or African Green, *Chlorocebus aethiops*) tend to be more sensitive to MPTP administration than New World monkeys (such as the squirrel monkey, *Saimiri sciureus* or marmoset, *Callithrix jacchus*) (Gerlach and Reiderer, 1996; Ovadia *et al.*, 1995; Rose *et al.*, 1993). Also, within a species, younger animals tend to be more resistant to the effects of MPTP and are more likely to recover from mild lesions compared to older animals (Collier *et al.*, 2007; Ovadia et al., 1995). There are a number of different reasons that account for the variability in MPTP sensitivity including its metabolism (especially in the liver), ability to cross the blood brain barrier, conversion of MPTP to the toxic form $MPP^+$ in the brain primarily by astrocytic monoamine oxidase B, uptake into dopaminergic neurons through the dopamine transporter, and distribution within cells to target mitochondrial energy depletion and to mediate cell death (Dauer and Przedborski, 2003; Jackson-Lewis and Smeyne, 2005).

**The Subtypes of the MPTP-Lesion Nonhuman Primate Model**

Administrating MPTP through a number of different regimens has led to the development of several distinct models of parkinsonism in the nonhuman primate that vary in both behavioral and pathological features. Each model is characterized by unique behavioral and neurochemical features, which should be taken into consideration when addressing specific scientific objectives or designing studies. As a result, numerous studies addressing a variety of hypothesis have been carried out in these different models. These studies include the investigation of basal ganglia function including motor behavioral recovery, mechanisms of motor complications, and cognitive impairment. These models also help in the evaluation of new treatment modalities including pharmacological agents, cell transplantation, deep brain stimulation, and novel neuroprotective and restorative strategies. In some models (such as the intracarotid delivery of MPTP) there is profound striatal dopamine depletion and denervation with few or almost no dopaminergic axons or terminals remaining. This model provides an optimal setting to test fetal tissue grafting since the presence of any tyrosine hydroxylase positive axons or sprouting cells would be due to surviving transplanted tissue or its influence on intrinsic striatal neurons. Other models, such as mild systemic delivery of MPTP, have less extensive dopamine depletion and only partial denervation with a moderate number of dopaminergic axons and terminals remaining. This partially denervated model best resembles mildly to moderately affected PD patients. Therefore, sufficient dopaminergic neurons and axons as well as compensatory mechanisms are likely to be present. Growth factors (inducing sprouting) or neuroprotective factors (promoting cell survival) are best evaluated in this situation. The most commonly used MPTP-lesioning paradigms in nonhuman primate models include; (1) the systemic-lesioned model; (2) hemi-lesioned; (3) bilateral intracarotid; (4) over-lesioned; and (5) low-dose chronic. The following sections briefly highlight the features of these different models. It should be kept in mind that these models are delineated based on the mode of MPTP delivery rather than the species of nonhuman primate utilized. Other factors will influence these models due to the degree of lesioning including the age, subspecies, and sex of the animals used.

(1) In the ***systemic lesioned model***, MPTP is administered via intra-muscular, intra-venous, intra-peritoneal, or subcutaneous injection (Eidelberg *et al.*, 1986; Elsworth *et al.*, 1990; Tetrud and Langston, 1989; Waters *et al.*, 1987). This model is most common with smaller nonhuman primates such as the squirrel monkey and marmoset, which can tolerate modest levels of MPTP. Old World monkeys tend to display a high degree of sensitivity towards MPTP and if utilizing systemic administration small doses are typically used and are spread out over several weeks. Large doses result in severe akinesia and may require intensive

veterinarian intervention including prolonged hand feeding to rescue animals. In addition, large doses can result in increased risk of animal death from the effects of MPTP and $MPP^+$ on peripheral organs. Systemic administration of MPTP leads to bilateral depletion of striatal dopamine and nigrostriatal cell death. One feature of this model is that the degree of lesioning can be titrated by adjusting the MPTP concentration administered resulting in a range (mild to severe) of parkinsonian symptoms. The presence of clinical asymmetry in motor features is common with one side more severely affected, but this feature may be subtle. Levodopa or dopamine agonist administration leads to the reversal of all behavioral signs of parkinsonism in a dose-dependent fashion. After several days to weeks of levodopa administration, animals develop reproducible motor complications. The principal advantage of this model is that the behavioral syndrome closely resembles the clinical features of idiopathic PD. The systemic model has partial dopaminergic denervation bilaterally and probably best represents the degree of loss seen in all stages of PD including end-stage disease, where some dopaminergic neurons are still present. This model is well suited for therapeutics that interact with remaining dopaminergic neurons including growth factors, neuroprotective agents, and dopamine agonists. The easily reproducible dyskinesia in this model allows for extensive investigation of its underlying mechanism and treatment (see below). Disadvantages of this model include spontaneous recovery in mildly affected animals, while severely affected animals may require extensive veterinary care and dopamine supplementation, specifically in the early period following MPTP-lesioning. In most cases early interventive care is necessary to overcome the systemic effects of MPTP on peripheral organ systems including the liver, kidneys, and heart which are typically transient (see section on systemic effects of MPTP below).

(2) The ***hemi-parkinsonian or hemi-lesioned model*** involves administration of MPTP via unilateral intracarotid infusion and has been used to induce a hemi-parkinsonian state in the primate (Bankiewicz *et al.*, 1986). The surgical delivery of MPTP goes directly to the brain, avoiding the systemic effects of MPTP as well as potential xenobiotic metabolism in the liver influencing its bioavailability. The rapid metabolism of MPTP to $MPP^+$ in the brain may account for the localized toxicity to the hemisphere ipsilateral to the infusion and in some cases can induce stroke-like lesions and necrosis within the basal ganglia (Emborg *et al.*, 2006). Motor impairments appear primarily on the contralateral side. Hemi-neglect, manifested by a delayed motor reaction time, also develops on the contralateral side. In addition, spontaneous ipsilateral rotation may develop. Levodopa administration reverses the parkinsonian symptoms and induces contralateral rotation. SNpc neurodegeneration and striatal dopamine depletion (greater than 99%) on the ipsilateral side to the injection is more extensive than in the systemic model. The degree of unilateral lesioning in this model is dose-dependent. Major advantages of this model include; (i) the ability of animals to feed and maintain themselves without supportive care; (ii) the availability of the relatively unaffected limb on the ipsilateral side to serve as a control; and (iii) the utility of the dopamine-induced rotation for pharmacological testing. In addition, due to the absence of dopaminergic innervation in the striatum, the hemi-lesioned model is well suited for examining neuronal sprouting of transplanted cells or tissue. A disadvantage of this model is that only a subset of parkinsonian features are evident and are restricted to one side of the body, a situation never seen in idiopathic PD.

(3) The ***bilateral intracarotid model*** employs an intracarotid injection of MPTP followed several months later by another intracarotid injection on the opposite side (Smith *et al.*, 1993). This model combines the less debilitating features of the carotid model with bilateral clinical features, a situation more closely resembling idiopathic PD. The advantage of this model is its prolonged stability and limited inter-animal variability. Similar to the hemi-lesioned model, where there is extensive striatal dopamine depletion and denervation, the bilateral intracarotid model is well suited for evaluation of transplanted tissue or vector infusion. However, levodopa administration may result in only partial improvement of parkinsonian motor features and food retrieval tasks. This can be a disadvantage since high doses of test drug may be needed to demonstrate efficacy, increasing the risk for medication related adverse side effects.

(4) The ***over-lesioned model*** is a novel approach to MPTP lesioning and involves the administration of MPTP via intracarotid infusion followed by a systemic MPTP injection (Eberling *et al.*, 1998). This model is characterized by severe dopamine depletion ipsilateral to the MPTP-carotid infusion and a partial depletion on the contralateral side due to the systemic MPTP injection. Consequently, animals are still able to maintain themselves as they retain one relatively intact side. The behavioral deficits consist of asymmetric parkinsonian features. The more severely parkinsonian side is contralateral to the intracarotid injection. Levodopa

produces a dose-dependent improvement in behavioral features. The complications of levodopa therapy, however, such as dyskinesia have not been as consistently observed. This model combines some of the advantages of both the systemic and intra-carotid MPTP models, including stability. This model is suitable for both transplant studies, utilizing the more depleted side, and neuro-regeneration with growth factors, utilizing the partially depleted side where dopaminergic neurons still remain.

(5) The ***chronic low dose model*** consists of intravenous injections of very low dose of MPTP administration over a five to thirteen month period (Bezard *et al.*, 1997; Decamp and Schneider, 2004; Slovin *et al.*, 1999a). Rather than primarily targeting motor behavior this model is characterized by cognitive deficits consistent with frontal lobe dysfunction reminiscent of PD or normal aged monkeys. These animals have impaired attention and short-term memory processes and perform poorly in tasks of delayed response or delayed alternation. Since gross parkinsonian motor symptoms are essentially absent at least in the early stages, this model is well adapted for studying cognitive deficits analogous to those that accompany idiopathic PD.

**The Adverse Effects of MPTP-Lesioning**

Apart from motor effects, MPTP clearly has central nervous system effects that may include hypothermia and seizures. The systemic administration of MPTP may also lead to deleterious peripheral effects that must be taken into consideration when designing therapeutic studies since they could lead to adverse effects influencing animal behavior, alterations in drug bioavailability, or alterations in drug-target interactions. For example, the peripheral conversion of MPTP to $MPP^+$, especially in the liver, can result in short-term changes in liver metabolism. Alterations within the liver can influence further MPTP administration sessions (especially those within the first week) and may alter xenobiotic metabolism of MPTP, $MPP^+$, or a therapeutic drug of interest. This point is illustrated in Figure 1. The level of serum $MPP^+$ was determined by HPLC analysis after each of 6 successive s.c. MPTP injections in the squirrel monkey. Data collected at post-lesioning day 1, 4, and 10 after each injection demonstrates altered serum $MPP^+$ levels indicating the peripheral conversion of MPTP to $MPP^+$ is occurring at a higher rate with later injections. Consequently, less MPTP is getting into the brain resulting is reduced lesioning with later injections. Our analysis of brain tissue early in the injection regimen shows that the majority of MPTP-induced cell loss occurs within the first 3 injections.

Systemic insult in the nonhuman primate may also influence motor and non-motor behavior, independent of any brain lesion, since animals that are sick by toxin may become very quiescent and disengaged. Furthermore, the peripheral effects on systemic organs, especially the heart, kidneys, and liver, are often responsible for the death of animals following MPTP administration. Death can occur within hours of MPTP administration indicating an immediate organ failure, or within the first week due to inability of animals to eat or drink by themselves. In addition, animals can become cachectic, and can show general wasting and decline without proper intervention. Supportive intervention can involve feeding lesioned nonhuman primates by gavage with an enriched diet, injection of subcutaneous fluids, or the administration of levodopa to promote movement. One must be cautious in introducing dopamine replacement therapy, since this may alter experimental outcome measures.

To address the potential adverse effects of MPTP, we studied issues of animal care that would result in greater animal survival. Squirrel monkeys were administered MPTP (in a series of 6 subcutaneous injections of 2 mg/kg, free-base, 2 weeks apart) and were given a comprehensive physical examination 1, 4, and 10 days after each injection. The results are summarized in Table 1. MPTP induces significant alterations in a number of physiologic parameters. Elevated liver transaminases indicated liver damage and elevated creatinine phosphokinase indicated muscle breakdown. By the second injection, there was a significant decrease in body weight, which was cumulative with each subsequent injection and tended not to recover without intervention. Greater than 20% loss of body weight is a significant predictor of death. Evidence of hepatocellular toxicity persisted for several weeks after the last injection. In addition, animals had hypothermia beginning 48 hours after lesioning and persisting for up to 10 days after the last MPTP injection. The pathophysiology of these effects may be directly related to MPTP itself and/or its metabolites and their adverse effects may persist for several weeks after the last injection. Overall, body weight and white blood cell count were the key predictors of mortality and should be monitored during MPTP administration. Supplemental caloric intake may be helpful

in improving survival. These studies highlight the systemic effects of MPTP on animal models that should be taken into consideration during the design of any pharmacological study. Researchers interested in details of MPTP toxicity and safety may refer to specific technical reviews (Jackson-Lewis and Przedborski, 2007; Przedborski *et al.*, 2001)

**Testing Pharmacological Therapies for Symptomatic Benefit**

Nonhuman primate models are especially useful for evaluating new symptomatic treatments of PD. In the past, the primary focus of experimental design has been on motor features, but some studies have targeted cognition and affective disorders. Typically, the experimental design evaluates the affect of an intervention, such as a dopamine agonist, on the animal's parkinsonian features. Many experimental pharmacological studies may utilize a colony of animals that have been used in several pharmacological studies and if drug exposure results in long-lasting effects it may influence subsequent studies. The time point when the MPTP-lesioning process itself is carried out should not interfere with the evaluation of the intervention must also be taken into consideration. Initiating intervention when animals are parkinsonian due to midbrain dopaminergic cell loss and/or striatal dopamine depletion is critical. Because the motor deficits can significantly recover over time in some models, it is important to include lesion-only controls to assess changes due to the dynamics of the model itself. When performing neuroprotective studies, another critical factor is the time of initiating an intervention with respect to MPTP-lesioning. For example, if an intervention is started before or during the lesioning phase, it may show evidence, suggesting neuroprotection from toxin-induced cell death. This neuroprotection may be due to the attenuation of cell death by directly supporting midbrain dopaminergic neurons to promote survival (as with neurotrophic factors), or it may be due to reduced insult to the brain by altering the bioavailability of the lesion-inducing toxin (i.e. decreasing the bioavailability of $MPP^+$). For example, if a drug suppresses the conversion of MPTP to $MPP^+$ by inhibiting monoamine oxidase B, then less $MPP^+$ will be generated leading to a reduced level of cell death or injury. We are beginning to appreciate that a number of aspects of the lesioning process may be altered by either pharmacology or behavior interventions. One mechanism could involve the expression or function of the dopamine transporter (DAT), the primary uptake site for both $MPP^+$ and 6-OHDA. If down-regulated, less neurotoxin will be taken up by dopaminergic neurons leading to reduced lesioning. Another mechanism could involve the expression of the vesicular monoamine transporter-2 (VMAT-2), with over-expression leading to increased sequestration of $MPP^+$ into vesicles, again leading to a smaller lesion.

Starting an intervention after neurotoxin-induced cell death is complete will permit evaluation of either symptomatic or neurorestorative treatment. For example, testing of novel dopamine agonists or compounds that target other neurotransmitter systems such as adenosine or glutamate may provide symptomatic benefit by enhancing dopamine neurotransmission either directly (by acting on dopamine receptors themselves) or indirectly (by affecting other dopaminergic parameters such as release or downstream effector pathways). Neurorestoration involves a different approach where an intervention such as gene delivery via stereotactic vector targeting or the transplantation of genetically engineered or stem/progenitor cells is intended to result in permanent benefit by replacement of lost basal ganglia function. The different models provide contrasting templates to evaluate such interventions. The systemic lesion model typically results in some residual degree of midbrain dopaminergic neuron survival. The surviving dopaminergic neurons in the systemic models will have some degree of striatal innervation despite severe dopamine depletion (Petzinger *et al.*, 2006) but could serve as a template where surviving dopaminergic neurons and their striatal projections may be promoted to recover. These surviving cells and their axonal projections may serve as substrates for neurotrophic factors such as glia derived neurotrophic factor (GDNF) (Kordower *et al.*, 2000; Oiwa *et al.*, 2006). In contrast, the intra-carotid lesion models, analogous to the 6-OHDA-lesoined rat, have a near complete depletion of nigrostriatal dopaminergic neurons and their axonal projections. In this model, an analogous neuronal template may be absent but the basal ganglia may serve as a "blank slate" where the transplantation of stem/progenitor cells can be evaluated (Sortwell *et al.*, 1998; Taylor *et al.*, 1991). More detail of these approaches can be found in other related chapters in this book.

The MPTP-lesioned nonhuman primate model can also help us better understand the potential properties of pharmacological treatments already in clinical use. For example, an interest in our laboratory is to elucidate the underlying mechanisms of intrinsic motor recovery in the squirrel monkey following systemic lesioning with MPTP. It is hypothesized that dopamine could, in fact, act as a neurotrophic factor helping to

maintain the integrity of the basal ganglia (Borta and Hoglinger, 2007). We were interested in knowing if dopamine replacement therapy with either levodopa or a dopamine agonist could provide benefit beyond purely symptomatic improvement. In a set of experiments performed in our labs, MPTP-lesioned squirrel monkeys treated with the dopamine agonist pramipexole, and to a lesser extent those treated with levodopa, had higher levels of striatal dopaminergic markers (levels of dopamine, tyrosine hydroxylase and dopamine transporter proteins) and amphetamine-induced dopamine release than those treated with saline. This occurred despite similar degrees of cell loss based on SNpc counts. These data suggest that dopamine replacement therapy can have a beneficial effect not only on symptomatic treatment of parkinsonian features but also may influence neuroplasticity in the injured basal ganglia. Pharmacological treatment of motor symptoms targeting dopamine replacement may have an analogous effect in patients with PD.

**Dyskinesia and Motor Complications in Nonhuman Primates**

In PD, levodopa therapy in many patients leads to the development of motor complications, typically after a few years. The underlying pathogenesis of these complications remains obscure (Blanchet *et al.*, 2004; Brotchie, 2005; Jenner, 2003b; Vitek and Giroux, 2000). For levodopa-induced dyskinesias, abnormal involuntary movements induced by levodopa, studies have implicated several neurotransmitter systems, especially the dopaminergic and the glutamatergic systems. As in patients with PD, the parkinsonian nonhuman primate also develops complications of levodopa therapy, with both motor fluctuations (wearing-off) and levodopa-induced dyskinesias. In this model, the animals develop movements that are abnormal (i.e., they differ phenomenologically from movements that are typically present), but it is impossible to determine whether they are involuntary since we cannot ask the animal its intent in making the movements. We can only judge whether the movements appear purposeful and use this to infer the degree of voluntary control. In any case, the movements bear a striking resemblance to levodopa-induced dyskinesias in patients. For example, dyskinetic movements in the MPTP-lesioned squirrel monkey or marmoset involve all four limbs and the trunk, with choreoathetoid movements that develop a few minutes after a dose of levodopa and last 3 to 4 hours. The time course for the movements corresponds to the time course for reversal of MPTP-induced bradykinesia. Observation of the phenomenology of the movements has led to rating scales that have been developed by a number of different groups for squirrel monkeys, marmosets, and macaques (Brotchie, 1999; Chassain *et al.*, 2001; Petzinger *et al*., 2001; Tan *et al*., 2002).

The effect of pharmacologic agents upon levodopa-induced dyskinesias have been studies for a variety of agents, including D2-dopamine receptor agonists (Calon *et al.*, 1995; Smith *et al.*, 2006), D3-dopamine receptor partial agonists (Hsu *et al*., 2004; Smith *et al*., 2006), dopamine receptor antagonists (Andringa *et al.*, 1999), A2a-adenosine receptor antagonists (Blandini, 2003; Kanda *et al.*, 1998), opioid receptor agents (Cox *et al.*, 2007; Fox *et al.*, 2002; Samadi *et al.*, 2003, 2004), and glutamate receptor antagonists (Papa and Chase, 1996; Samadi *et al.*, 2007; Verhagen-Metman *et al.*, 1998). Unfortunately, the search for an effective suppressor of levodopa-induced dyskinesias has had limited success, although some studies have provided clues for a useful therapeutic strategy – minimizing wide fluctuations in the delivery of levodopa to the striatum. Although the underlying mechanism of levodopa-induced dyskinesias is unknown, electrophysiological, neurochemical, molecular, and neuro-imaging studies in the nonhuman primate models suggest that the pulsatile delivery of levodopa may lead to a variety of changes in the postsynaptic cell and in other parts of the basal ganglia that are further downstream, giving rise to levodopa-induced dyskinesias. These changes could include; (i) changes in the neuronal firing rate and pattern of the globus pallidus and subthalamic nucleus; (ii) enhancement of D1- and/or D2- receptor mediated signal transduction pathways; (iii) super-sensitivity of the D2 receptor; (iv) alterations in the phosphorylation state or subcellular localization of glutamate receptors; (v) modifications in dopamine receptor subtypes and their functional links; and (vi) enhancement of opioid-peptide mediated neurotransmission (Bedard *et al.*, 1992; Bezard *et al.*, 2001; Calon *et al.*, 2002; Hurley *et al.*, 2001; Papa and Chase, 1996).

In designing studies to examine potential treatments for suppressing levodopa-induced dyskinesias, it is necessary to examine the effect on both the dyskinesias and the parkinsonism, as mentioned above. A drug that improves dyskinesias at the expense of worsening parkinsonism will be of limited utility as a treatment in patients. It is also important to keep in mind that dyskinesias differ phenomenologically and possibly mechanistically in different primate species. For example, such a difference might exist for the occurrence of

facial dyskinesias, which have been observed in Old World but not New World monkeys (Petzinger *et al*., 2001; Tan *et al*., 2002).

The presence of a nigral lesion has long been considered a necessary prerequisite for the development of levodopa-induced dyskinesias with the intensity dependent on the degree of lesioning (Di Monte *et al.*, 2000; Kuoppamaki *et al.*, 2007; Schneider *et al.*, 2003). Recent studies have challenged this dogma and it has been reported that nonhuman primates without any dopaminergic lesions can manifest levodopa-induced dyskinesias. For example, when administered sufficiently large doses of levodopa, dyskinesias can develop in squirrel monkeys within a few days (Togasaki *et al.*, 2001), and in marmosets within 8 weeks (Pearce *et al.*, 2001). The relatively high doses of levodopa administered to these animals may serve to exhaust the buffering capacity of the dopaminergic terminals within the striatum and accentuate the pulsatile deliver of dopamine to the postsynaptic receptors of the normal animal, thus giving rise to dyskinesias.

**Intrinsic Neuroplasticity and Behavioral Recovery**

Understanding the molecular mechanisms underlying behavioral recovery in the nonhuman primate may provide insights into neuroplasticity of the brain after injury, help identify new therapeutic targets for treatment of PD, and provide an opportunity to elucidate basal ganglia function (Bezard and Gross, 1998; Jakowec *et al.*, 2003; Jakowec *et al.*, 2004; Zigmond, 1997; Zigmond *et al.*, 1990). Behavioral recovery after MPTP-induced parkinsonism has been reported in both New and Old World nonhuman primates (Cruikshank and Weinberger, 1996; Eidelberg *et al*., 1986; Kurlan *et al.*, 1991; Oiwa *et al.*, 2003; Petzinger *et al*., 2006; Petzinger and Langston, 1998; Schneider *et al.*, 1995; Schneider *et al.*, 1994; Scotcher *et al.*, 1991). The degree and time course of behavioral recovery is dependent on age, species, and mode of MPTP administration (Albanese *et al.*, 1993; Ovadia et al., 1995; Petzinger and Langston, 1998; Taylor *et al.*, 1997). In general, severely lesioned animals are less likely to recover than mildly lesioned animals, and intracarotid injection models are less likely to recover than systemic models (Taylor *et al*., 1997). For example, squirrel monkeys rendered severely parkinsonian by a series of 6 subcutaneous injections of MPTP over a 12-week period recover motor behavior within several months of their last injection (Petzinger *et al.*, 2006). Figure 2 demonstrates differences in the time course of recovery of motor behavior depending on the degree of MPTP-lesioning. In contrast to systemic MPTP-lesioning, nonhuman primates administered MPTP via intra-carotid injection tend not to recover motor behavior and may display stable parkinsonian features for months to years after lesioning. These differences in recovery between the various models likely reflect differences in the degree of midbrain dopaminergic cell loss. With systemic administration of MPTP, a proportion of SNpc dopaminergic neurons are spared (between 40 and 60%), whereas in the intra-carotid paradigm almost no midbrain dopaminergic neurons remain ipsilateral to the site of injection. In fact, intra-carotid lesioning can be so intense as to cause necrosis and stroke in the ipsilateral striatum (Emborg *et al.*, 2006).

Studies investigating the mechanisms of recovery in these models have shown; (i) alterations in dopamine biosynthesis (tyrosine hydroxylase) and metabolism (increased turnover); (ii) altered regulation of dopamine transporter (DAT) expression and function; (iii) sprouting and branching of tyrosine hydroxylase fibers; (iv) alterations of other neurotransmitter systems including glutamate and serotonin; and (v) alterations of signal transduction pathways in both the direct (D1) and indirect (D2) pathways (Bezard and Gross, 1998; Chiueh *et al.*, 1984a; Cruz-Sanchez *et al.*, 1993; Eidelberg *et al*., 1986; Frohna *et al.*, 1995; Ho and Blum, 1998; Jakowec et al., 2004; Mitsumoto *et al.*, 1998; Morgan *et al.*, 1991; Mori *et al.*, 1988; Nishi *et al.*, 1989; Rose *et al.*, 1989; Rothblat *et al.*, 2001; Rozas *et al.*, 1998; Russ *et al.*, 1991; Wade *et al.*, 2001). Interestingly, the return of striatal dopamine is incomplete despite full motor recovery, although more pronounced return in the ventral striatum compared to the dorsal regions has been reported in several different MPTP-lesioned species (Elsworth *et al.*, 2000; Petzinger *et al.,* 2006). In the squirrel monkey we found that dopamine levels in tissue homogenate increased from 0.7% to 1.6% of baseline in the dorsal putamen at 6 weeks (when animals are moderately parkinsonian) and 9 months (when animals are fully recovered from motor impairment) (Petzinger *et al.*, 2006). However, in the ventral putamen dopamine levels at the same time points were 8.7% and 28.0% of baseline, respectively. One explanation may be that the ventral dopaminergic system is less sensitive to MPTP toxicity (Moratalla *et al.*, 1992). This raises the possibility that the ventral striatum, with a higher dopamine return than the dorsal, may play a role in behavioral recovery and one means may be through the diffusion of dopamine into the dorsal denervated regions (Schneider *et al.*, 1994). Even with insufficient total dopamine return, studies in the rodent models have shown that when dopamine loss is less than 80%,

homeostatic mechanisms can lead to complete normalization of extracellular levels of dopamine; however, when dopamine depletion exceeds 80% there is only partial normalization (Abercrombie *et al.*, 1990; Altar and Marien, 1989; Castaneda *et al.*, 1990; Robinson and Wishaw, 1988). In our studies we also observed a dynamic change in protein expression in the caudate nucleus and putamen, where animals at 9 months after lesioning compared to animals at 6 weeks, showed increased levels of tyrosine hydroxylase and DAT protein that was more dramatic in the ventral than dorsal regions of the basal ganglia. Studies suggest that there are many pre- and post-synaptic molecular changes that occur as a consequence of dopamine denervation/dysfunction which may contribute to behavioral recovery and/or play a role in other phenomenology such as susceptibility to levodopa-induced dyskinesias. The fact that there are molecular and neurochemical changes that occur in a time course fashion in these models underscores the importance of considering the time from lesioning as an important parameter in study design. Another cautionary point is that many of these molecular changes may take place in a time course fashion even in animals that do not display overt behavioral recovery. This raises the issue that studies should consider time since lesioning as a potential factor in influencing outcome measures when examining molecular and physiological changes.

**Electrophysiological Studies of Basal Ganglia Function the Nonhuman Primate Model of PD**

An important aspect of the nonhuman primate is the anatomical similarity of basal ganglia structure and function to that of humans, thereby providing an important tool for investigating basal ganglia function, such as neurophysiological properties in the normal and disease state, and thus serves as the foundation for identifying new therapeutic treatments. For example neurophysiological studies have implicated over-activity at corticostriatal synapses as one underlying mechanism for the development of motor impairment in PD (Konitsiotis *et al.*, 2000; Soares *et al.*, 2004; Wichmann and DeLong, 2003). Electrophysiological studies in our labs, using the MPTP-lesioned squirrel monkey, have shown changes in AMPA and GABA mediated synaptic neurotransmission that may account for excessive excitatory corticostriatal drive. For these studies, we administered MPTP in a series of 6 subcutaneous injections of 2.0 mg/kg (free-base) every 2 weeks for a total of 12 mg/kg. Whole brains were harvested at either 6 weeks (when animals are parkinsonian) or 9 months (when animals are motorically recovered) after the last injection of MPTP and striatal synaptic function was examined in coronal *in vitro* brain slices. We found that the input/output relationship was greater for AMPA receptor mediated synaptic currents at 6 weeks after MPTP-lesioning compared to saline control using whole cell voltage clamp. The relative strength of $GABA_A$ versus AMPA receptor mediated synaptic responses was calculated as the $I_{GABA-}$ changes in $_A$ / $I_{AMPA}$ ratio. Interestingly, we also found a reduced $I_{GABA-A}/I_{AMPA}$ ratio 6 weeks after MPTP. These GABAergic inhibition that we and others have observed may play an important role in facilitating the synchrony and oscillatory patterns of discharge found throughout the basal ganglia motor circuit in MPTP-treated akinetic primates (Goldberg *et al.*, 2002; Raz *et al.*, 1996; Raz *et al.*, 2001). Analysis of animals 9 months after MPTP administration suggests there is normalization of corticostriatal hyperactivity when animals demonstrate full behavioral recovery. Specifically we found the input/output ratio for AMPA receptor-mediated synaptic responses and the $I_{GABA-A}$ / $I_{AMPA}$ ratio returned back to control levels (Figure 3). These observations are in agreement with the view that excessive glutamatergic corticostriatal synaptic function may be a contributing factor to the behavioral pathology of PD (Konitsiotis *et al.*, 2000; Muriel *et al.*, 2001). Future studies will exam whether changes in glutamatergic drive in fully recovered animals differentially impacts corticostriatal synapses in direct versus indirect basal ganglia pathways, as has been reported in the parkinsonian state (Day *et al.*, 2006; Wichmann and DeLong, 2003).

Dopamine denervation in animal models of PD is also associated with changes in the molecular composition of AMPA and NMDA receptors in the striatum (Betarbet *et al.*, 2004; Betarbet *et al.*, 2000; Hallett *et al.*, 2005; Hurley *et al.*, 2005; Nash *et al.*, 2004). We also found evidence for changes in the pharmacological profile of AMPA and NMDA receptors, which are consistent with these molecular studies. For example as shown in Figure 3, in animals examined 6 weeks post MPTP-lesioning, we found; (i) a decrease in the $I_{NMDA}/I_{AMPA}$ ratio; (ii) an alteration in the NMDA receptor subunit composition as indicated by increased sensitivity to the selective NR2B antagonist CP-101,606; and (iii) an alteration in AMPA receptor mediated synaptic responses, as indicated by changes in the sensitivity to the selective AMPA receptor antagonist, GYKI-52466 compared to saline control animals (Nash *et al*., 2004; Ruel *et al.*, 2002). Again, with behavioral recovery at 9 months post-MPTP-lesioning, we observed the trend of a return of NMDA and AMPA receptor function to match that seen in saline injected squirrel monkeys (Figure 3).

The glutamatergic corticostriatal and the dopaminergic nigrostriatal system are important mediators of synaptic plasticity, termed long-term depression (LTD) and long-term potentiation (LTP), within the basal ganglia (Centonze *et al.*, 2001; Mahon *et al.*, 2004; Picconi *et al.*, 2005; Reynolds and Wickens, 2002). Electrophysiological studies in our lab, using saline control squirrel monkeys, have shown that the induction of long-term synaptic plasticity at corticostriatal synapses is region specific, with LTP being induced in more medial regions and LTD in more lateral regions. These findings agree with previous reports from the rodent model of PD (Partridge *et al.*, 2000; Smith *et al.*, 2001). Studies in the rat have shown a loss of synaptic plasticity after 6-OHDA administration, which we have observed in the MPTP-lesioned mouse model, 1 to 2 weeks after neurotoxicant exposure (Calabresi *et al.*, 1992; Centonze *et al.*, 1999; Kreitzer and Malenka, 2007). Presently, there is little known regarding alterations in synaptic plasticity immediately following MPTP lesioning in the nonhuman primate.

Analysis of the expression of synaptic plasticity in the squirrel monkey 9 months after MPTP-lesioning has shown that LTD and LTP expression is evident. In the same animals used for analysis of glutamate neurotransmission above, we observed a dramatic and permanent decrease in dopamine release as measured by fast-scan cyclic voltammetry (Cragg, 2003) (Figure 4). This finding is in agreement with previous reports examining dopamine function in the squirrel monkey using HPLC (Petzinger *et al*., 2006). The expression of dopamine-dependent forms of LTP we observed in the dopamine depleted squirrel monkey suggest an adaptation may occur in the expression and/or sensitivity of both D1 and D2 receptors (Centonze *et al*., 2001; Mahon *et al*., 2004; Picconi *et al.*, 2005; Reynolds and Wickens, 2002). Preliminary studies in our lab have shown that LTD expression at lateral cortico-putamen synapses from the 9-month MPTP-lesioned squirrel monkey is D2 dependent, since this effect is blocked by the D2 receptor antagonist *l*-sulpiride. In addition, use of *l*-sulpiride results in the unexpected expression of LTP in lateral synapses (Figure 4). Our findings are consistent with the literature, where dopamine receptors D1 and D2 have been shown to play an important role in LTP and LTD, respectively (Calabresi *et al.,* 1992; Centonze *et al.*, 1999; Wang *et al.,* 2006). Taken together, these data suggest behavioral recovery from MPTP exposure in the squirrel monkey may be due at least in part to compensatory increases in the sensitivity of dopamine receptors, which enables the normal and expected expression of long-term plasticity at corticostriatal synapses.

**6-Hydroxydopamine**

6-Hydroxydopamine (6-OHDA or 2,4,5-trihydroxyphenylethylamine) is a specific catecholaminergic neurotoxin structurally analogous to both dopamine and noradrenalin. Acting as a “false-substrate”, 6-OHDA is rapidly accumulated in catecholaminergic neurons. The mechanism of 6-OHDA toxicity is complex and involves; (i) alkylation; (ii) rapid auto-oxidization (leading to the generation of hydrogen peroxide, superoxide, and hydroxyl radicals); and (iii) impairment of mitochondrial energy production (Blum *et al.*, 2001; Glinka and Youdim, 1997). The 6-OHDA induced rat model of PD was initially carried out by Ungerstedt in 1968, using stereotaxic bilateral intracerebral injections into the substantia nigra or lateral hypothalamus (targeting the medial forebrain bundle) (Ungerstedt, 1968). In addition to the rat, other species including the nonhuman primate (specifically the marmoset) have served as models for 6-OHDA lesioning (Annett *et al.*, 1992; Eslamboli, 2005). Lesioning in nonhuman primates provides for the analysis of behaviors not observed in the rat, such as targeting and retrieval tasks of the arm and hand. This model, however, has not gained popularity for nonhuman primates because the toxin must be delivered directly in the vicinity of the dopamine cells by intracerebral injections. This is much more difficult method than administering MPTP systemically.

**Methamphetamine**

Amphetamine and its derivatives (including methamphetamine, N-methyl-beta-phenylisopropylamine) lead to long lasting depletion of both dopamine and serotonin when administered to rodents and nonhuman primates including vervet, macaques, squirrel monkeys, and baboons (Czoty *et al.*, 2004; Davidson *et al.*, 2001; Ricaurte *et al.*, 1982; Ricaurte *et al.*, 1980; Villemagne *et al.*, 1998). Methamphetamine (METH), one of the most potent of these derivatives, is typically administered in a series of small intramuscular or oral doses from 0.1 to 2 mg/kg and leads to dose-dependent terminal degeneration of dopaminergic neurons in the caudate nucleus and putamen, nucleus accumbens, and neocortex. Despite the severe depletion of striatal dopamine, the motor behavioral alterations seen in rodents and nonhuman primates tend to be transient and subtle (Walsh and Wagner, 1992). The neurotoxic effects of METH are dependent on the efflux of dopamine since agents that deplete dopamine or block its uptake are neuroprotective (Fumagalli *et al.*, 1998; Westphale

and Stadlin, 2000). The metabolic mechanisms underlying METH-induced neurotoxicity involve the perturbation of antioxidant enzymes such as glutathione peroxidase or catalase leading to the formation of reactive oxygen/nitrogen species including $H_2O_2$, superoxide, and hydroxyl radicals (Cubells *et al.*, 1994; Davidson et al., 2001; Gluck *et al.*, 2001; Imam *et al.*, 2001; Yamamoto and Zhu, 1998). The administration of antioxidant therapies or over-expression of superoxide dismutase (SOD) in transgenic mouse models is neuroprotective against METH toxicity (Cadet *et al.*, 1994; Hirata *et al.*, 1996). In addition, both glutamate receptors and nitric oxide synthase (NOS) are important to METH-induced neurotoxicity since the administration of either NMDA receptor antagonists or NOS inhibitors are also neuroprotective (Sonsalla *et al.*, 1991). Other factors important to METH-induced neurotoxicity include the inhibition of both tyrosine hydroxylase and dopamine transporter activity and METH-induced hyperthermia (Imam *et al.*, 2001).

In contrast to MPTP, which destroys nigrostriatal dopaminergic neurons and their terminals, METH administration spares axonal trunks and soma of SNpc and VTA neurons targeting terminals found within the caudate nucleus and putamen (Kim *et al.*, 2000). Depending on the species and dosing regimen of METH, the effects of lesioning involves a spectrum from axonal degeneration to suppression of markers of nigrostriatal neuron integrity including tyrosine hydroxylase, DAT, and VMAT-2 proteins. The fact that these markers can be differentially affected by METH indicates that phenotypic suppression in dopaminergic neurons is a significant feature of METH exposure. There have been occasional reports of METH-induced cell death in the SNpc but they are subtle compared to the substantial levels seen with MPTP-induced cell death (Sonsalla *et al.*, 1996). In general, the effects of severe METH lesioning are long lasting. Interestingly, there is evidence of recovery of dopaminergic system depending on the METH regimen and species used (Harvey *et al.*, 2000a). Studies employing PET-imaging in conjunction with histological analysis of markers of the dopaminergic system have demonstrated that re-establishment of the nigrostriatal system occurs which probably involves a combination of re-innervation (neuronal sprouting) and return of previously suppressed TH and DAT protein expression (Harvey *et al.*, 2000b; Melega *et al.*, 1997).

Similar to studies with MPTP, METH administration demonstrates the dynamic neuroplasticity of the nigrostriatal system and its ability to respond to neurotoxic injury. The administration of METH to adult animals has played an important role in testing the molecular and biochemical mechanisms underlying dopaminergic and serotonergic neuronal axonal degeneration especially the role of free radicals and glutamate neurotransmission. Understanding these mechanisms has led to the testing of different neuroprotective therapeutic modalities. An advantage of the METH model over MPTP is that the serotonergic and dopaminergic systems can be lesioned *in utero* during the early stages of the development of these neurotransmitter systems. Such studies have indicated that there is a tremendous degree of architectural rearrangement that occurs within the dopaminergic and serotonergic systems of injured animals as they develop. These changes may lead to altered behavior in the adult animal (Frost and Cadet, 2000). An finally, in light of the fact that METH and other substituted amphetamines (including MDMA “ecstasy”) are major drugs of abuse in our society, animal models have provided a means to understand the mechanisms of brain injury with these toxic compounds and to determine the long-lasting effects of these drugs (McCann *et al.*, 1998; Paulus *et al.*, 2002) including if individuals who abuse METH are prone to develop parkinsonism (Guilarte, 2001).

**Proteasome Inhibition**

An important regulatory system within cells is the ubiquitin proteasome system (UPS), a large enzymatic complex involved in detoxification and degradation of ubiquitin tagged proteins (Betarbet *et al.*, 2005; Ross and Pickart, 2004). Inhibition of the UPS can lead to the inability to remove toxic protein moieties, accumulation of protein aggregates, neuronal dysfunction, and cell death (Petrucelli and Dawson, 2004; Tanaka *et al.*, 2004). The identification of genes involved in familial forms of parkinsonism especially parkin (an E3 ligase of UPS) and UCH-L1 (ubiquitin carboxy terminal hydrolase L1) have implicated a role of the UPS in idiopathic PD (Healy *et al.*, 2004; McNaught *et al.*, 2003; Petrucelli and Dawson, 2004; Tanaka *et al.*, 2004). Therefore, targeting the UPS through elevated oxidative stress, introduction of various gene mutants, or pharmacological targeting have been tested for developing parkinsonian features in both rodent and nonhuman primate models. The infusion of inhibitors of the UPS such as lactacystin and epoxymycin to the basal ganglia has been reported to result in the loss of TH and DAT immunoreactivity, dopamine depletion, and the occurrence of protein inclusions in midbrain dopaminergic neurons spared from cell death (Fornai *et al.*, 2003; McNaught *et al.*, 2004). Initial studies were carried out in the rodent but recent studies reported

analogous effects in the marmoset monkey (Eslamboli *et al*., 2007). However, the recent excitement in developing a new model for PD has been met with a degree of skepticism following reports from several labs that have not been able to replicate the initial findings targeting proteasome inhibition (Beal and Lang, 2006; Bove *et al.*, 2006; Kordower *et al.*, 2006; Manning-Bog *et al.*, 2006). Further evaluation by the scientific community will help evaluate the basis for these differences and to determine the potential utility of this model.

## Conclusion

To successfully translate findings in the laboratory to the clinical setting it is critical that novel experimental therapeutic approaches be evaluated in the nonhuman primate. Studies in normal animals can evaluate safety and tolerability issues, while studies in models of PD can test potential efficacy of pharmacological, surgical, and molecular approaches. Despite the fact that neurotoxicant models do not replicate all the pathological features seen in patients with PD, studies in nonhuman primate models can help us better understand the human condition by providing a template to test hypotheses. For example, understanding why a therapeutic approach shown to be efficacious in rodent models but fails in patients with PD can be addressed in nonhuman primates and may reveal previously unknown or unrecognized features of the disease (Jenner, 2003a,b). The nonhuman primate serves as an important link bridging the phylogenetic continuum between rodents and H*omo sapiens*. In finding new therapeutic modalities for the treatment of neurological disorders such as PD many researchers feel that the nonhuman primate provides an essential model to validate findings in the preclinical phase.

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## Legends

**Table 1: Systemic effects of MPTP in the nonhuman primate.** Squirrel monkeys received a series of 6 injections of MPTP (s.c., 2.0 mg/kg, free-base) with 2 weeks between injections. At days 1, 4, and 10 after each of the 6 MPTP injections animals were subjected to a comprehensive physical exam that included body weight, heart rate, blood pressure, core body temperature, blood cell counts, and comprehensive blood chemistry. Data are grouped according to either the MPTP exposure early (injections 1 to 3) or late (injections 4 to 6) as well as post-lesion time early (1 day) and late (10 days). These data demonstrate the variation in systemic parameters at different stage of lesioning.

**Figure 1: Altered levels of serum $MPP^+$ levels with successive injections of MPTP**. Squirrel monkeys were administered a series of 6 injections of MPTP (i.p., 2.0 mg/kg free-base, 2 weeks between each injection). Blood was collected at days 1, 4, and 10 after each injection of MPTP and the level of $MPP^+$ determined by HPLC analysis. These data demonstrate that with successive injections of MPTP there is an increase in the serum level of $MPP^+$ indicating systemic conversion of MPTP to $MPP^+$ most likely due to induction of metabolic enzymes in peripheral organs especially the liver. Since $MPP^+$ cannot cross the blood brain barrier the degree of lesioning in the brain is reduced with later injections of MPTP.

**Figure 2: Time course in motor recovery in the MPTP-lesioned nonhuman primate model**. Squirrel monkeys (N = 6 per group) were administered saline, 3 or 6 injections of MPTP (2.0 mg/kg free-base, 2 weeks between injections). A clinical rating scale (CRS) was administered to monitor parkinsonian features (Petzinger et al., 2006). A score greater than 4 is considered the threshold for parkinsonian motor features. Animals receiving 3 injections of MPTP displayed mild transient parkinsonian features for only a few weeks while those receiving 6 injections showed moderate to severe parkinsonian features and showed full motor behavioral recovery 12 weeks after the last injection of MPTP. The open and gray bars represent the time frame of 6 and 3 injections of MPTP, respectively.

**Figure 3: Electrophysiological evidence for MPTP-induced changes in synaptic transmission.**
**(A)** Input (stimulus intensity applied to corpus callosum) – output (excitatory postsynaptic current or EPSC amplitude) relationships were determined for cortico-putamen synapses using whole cell voltage clamp with $GABA_A$ receptor blocked by picrotoxin. Note a greater tendency for larger amplitude EPSCs at 6 weeks post-MPTP (left panel), with a return to normal amplitude EPSCs by 9 months post-MPTP (right panel).
**(B)** Example of synaptic currents recorded from a putamen neuron in response to corpus callosum stimulation before and after addition of picrotoxin. Synaptic currents were evoked in cells clamped at membrane potentials of –60 mV to maximize AMPA current activation and 0 mV to maximize $GABA_A$ current activation.
**(C)** Ratios of synaptic currents illustrate a shift in synaptic function. At 6-weeks post-MPTP there was a reduction in the $GABA_A$/AMPA ratio, a reduction in the NMDA/AMPA ratio, an increase in the NR2B/total NMDA ratio, and a decrease in the GYKI 52466 sensitive/CNQX sensitive AMPA ratio. Interestingly, these trends returned to saline control levels by 9 months post-MPTP.

**Figure 4: Changes in dopamine release and synaptic plasticity in the MPTP-lesioned nonhuman primate**.
**(A)** Fast-scan cyclic voltammetry revealed regional differences in evoked dopamine release in the putamen that is markedly reduced even after 9 months post-MPTP when animals are motorically recovered. Letters in the graph correspond to putamen brain slice sites.
**(B)** Comparison of short-term (3 min post-tetanus) and long-term (30 min post-tetanus) synaptic plasticity at corticostriatal synapses from saline and 9 post-MPTP lesioning. Intracellular recording of EPSPs evoked at cortico-putamen synapses was used to monitor changes in strength induced by tetanic activation of the corpus callosum. Medial cortico-putamen synapses produced short- and long-term potentiation (LTP) in both groups.

Lateral cortico-putamen synapses expressed short- and long-term depression (LTD) in saline and MPTP exposed monkeys, but the MPTP group tended toward greater LTD.
**(C)** Example of LTD induced at lateral cortico-putamen synapses and tetanic activation of lateral cortico-putamen synapses in the presence of the D2 receptor antagonist *l*-sulpiride that enabled the expression of profound LTP. Inset shows the response of the putamen neuron recorded during the *l*-sulpiride experiment to current injection, which is typical of a medium spiny projection neuron.

**Figure 1**

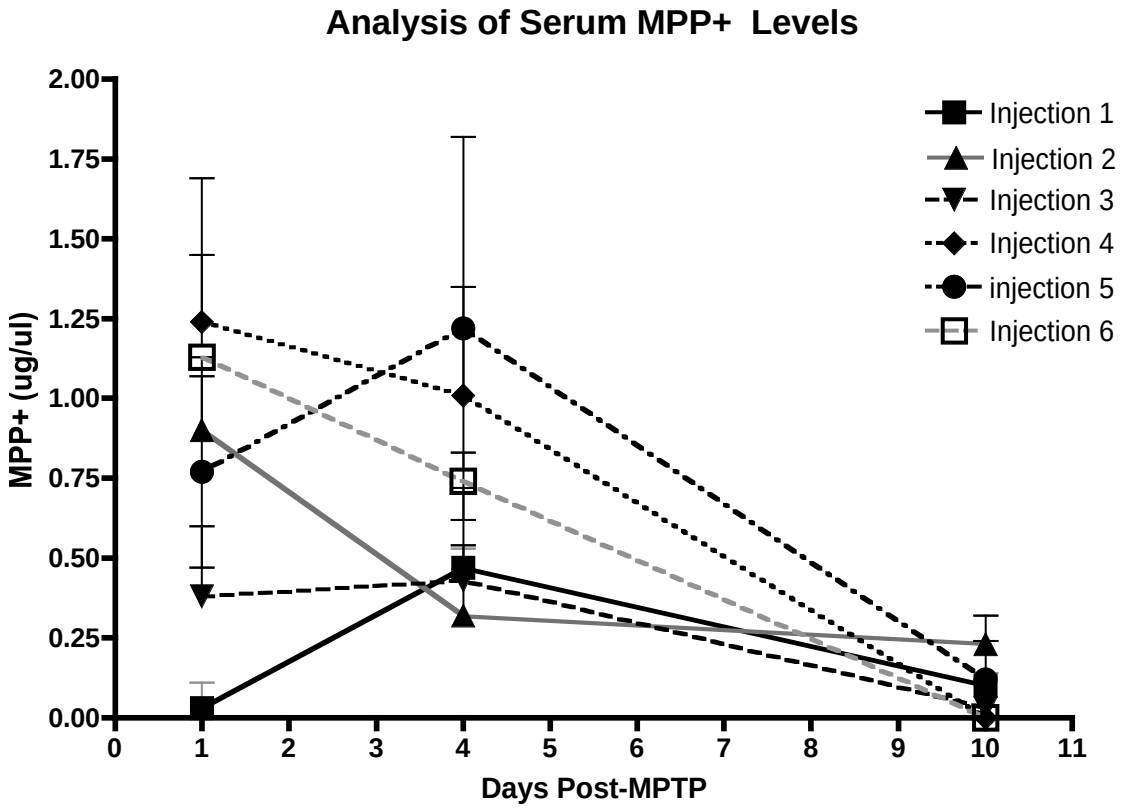

Analysis of Serum MPP+ Levels
MPP+ (ug/ul)
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
7
8
9
10
11
Days Post-MPTP
Injection 1
Injection 2
Injection 3
Injection 4
injection 5
Injection 6

Figure 2

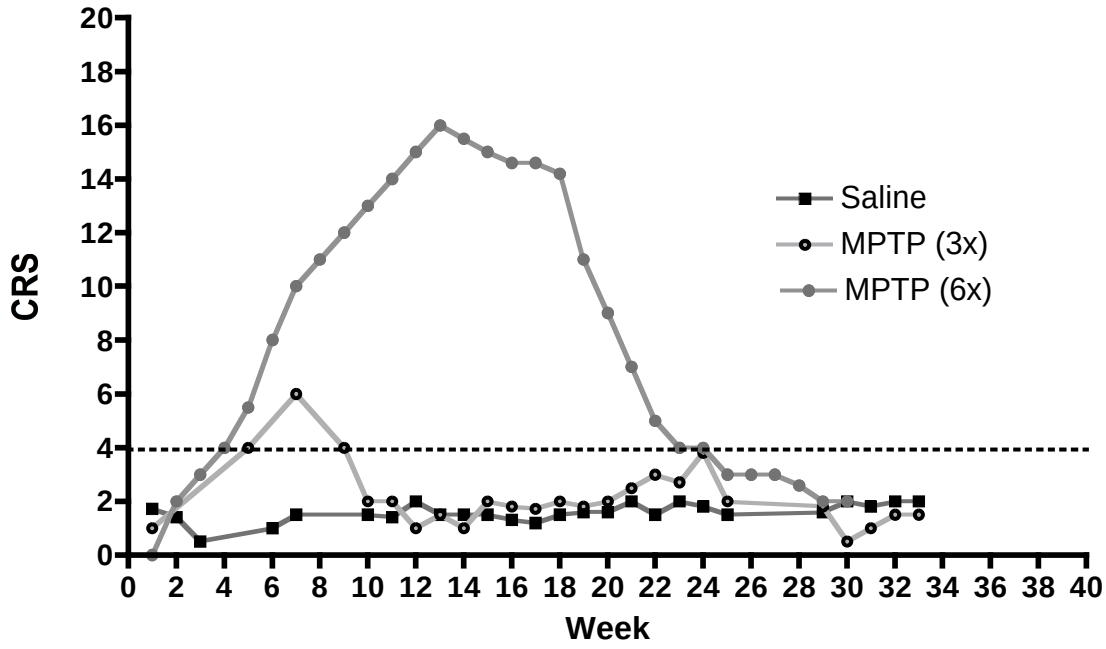

CRS
Week
Saline
MPTP (3x)
MPTP (6x)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40

Figure 3

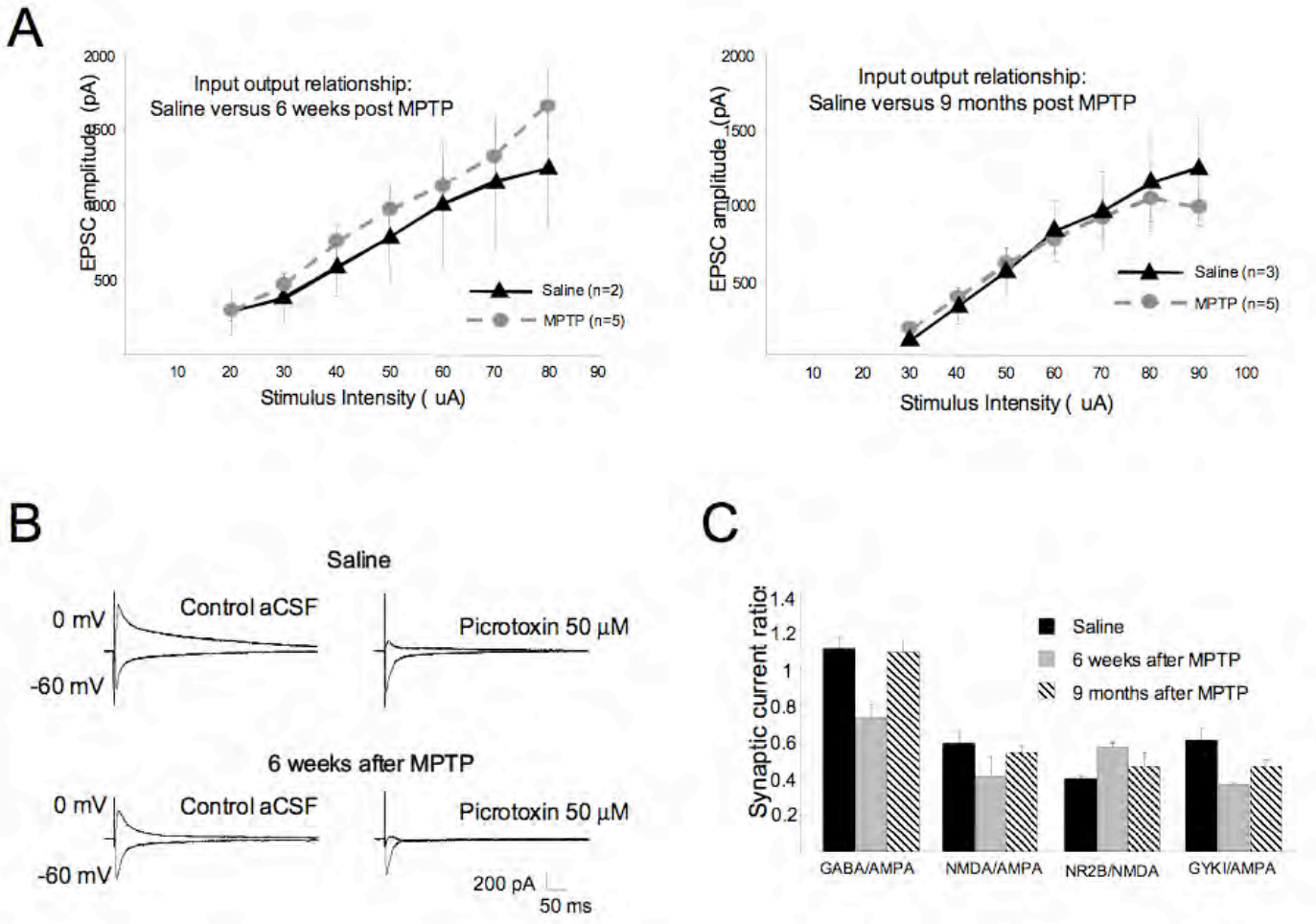
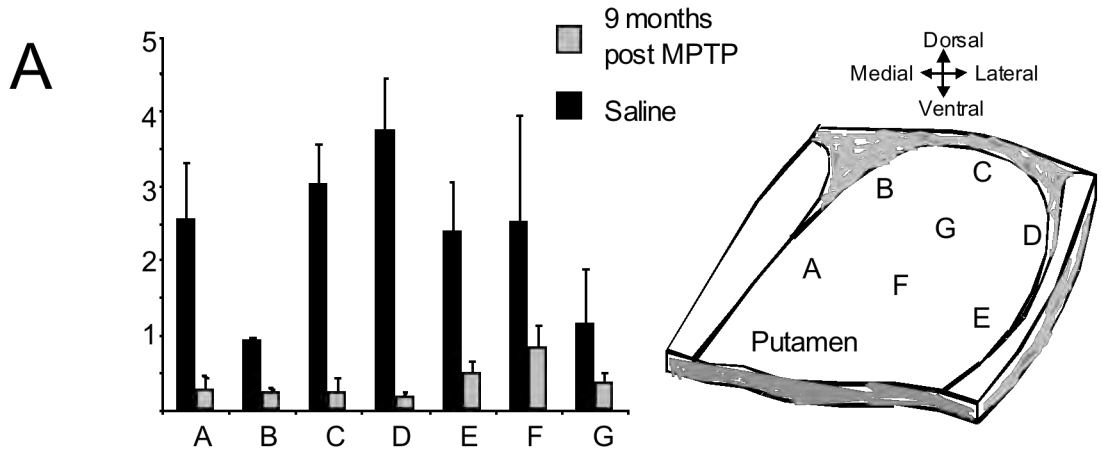
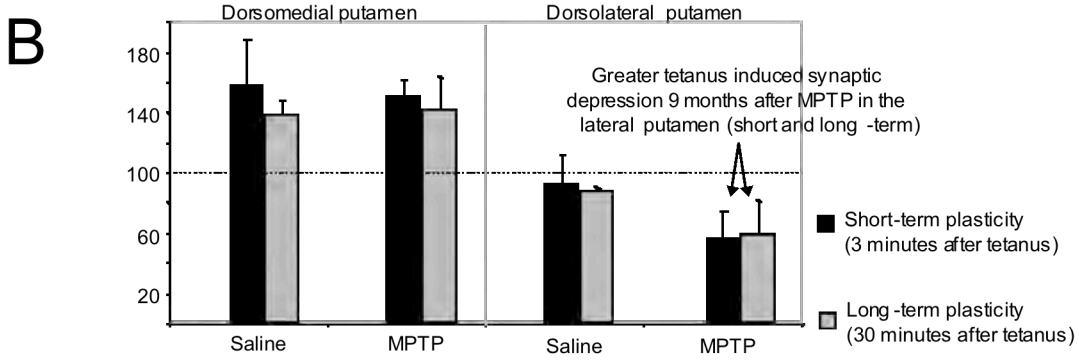
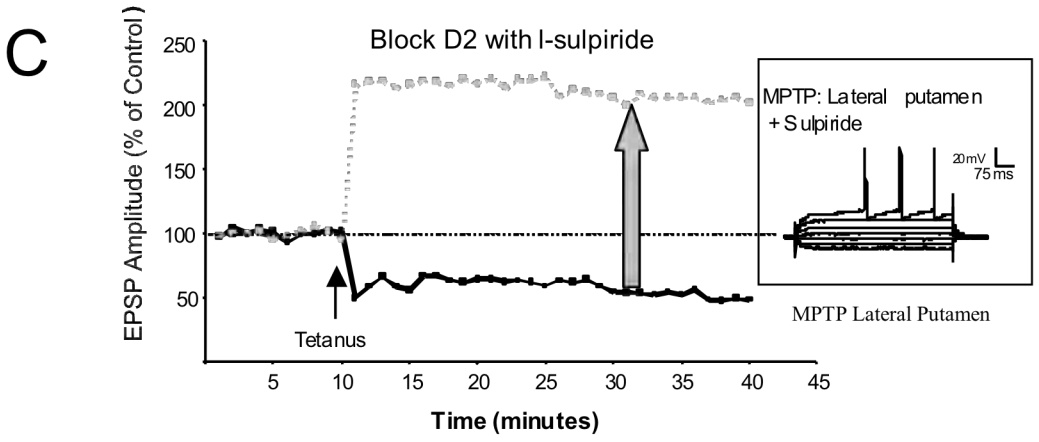

A
Input output relationship:
Saline versus 6 weeks post MPTP
EPSC amplitude (pA)
Stimulus Intensity ( uA)
Saline (n=2)
MPTP (n=5)
Input output relationship:
Saline versus 9 months post MPTP
Saline (n=3)
MPTP (n=5)
B
Saline
0 mV
-60 mV
Control aCSF
Picrotoxin 50 µM
6 weeks after MPTP
200 pA
50 ms
C
Synaptic current ratio
Saline
6 weeks after MPTP
9 months after MPTP
GABA/AMPA
NMDA/AMPA
NR2B/NMDA
GYKI/AMPA

Figure 4


A
9 months post MPTP
Saline
5
4
3
2
1
A B C D E F G
Dorsal
Medial
Lateral
Ventral
A
B
C
D
E
F
G
Putamen


B
Dorsomedial putamen
Dorsolateral putamen
180
140
100
60
20
Greater tetanus induced synaptic depression 9 months after MPTP in the lateral putamen (short and long -term)
Short-term plasticity (3 minutes after tetanus)
Long -term plasticity (30 minutes after tetanus)
Saline
MPTP
Saline
MPTP


C
Block D2 with l-sulpiride
EPSP Amplitude (% of Control)
250
200
150
100
50
MPTP: Lateral putamen + Sulpiride
20mV
75ms
MPTP Lateral Putamen
Tetanus
5 10 15 20 25 30 35 40 45
Time (minutes)